BIONOMICS OF MALARIA VECTORS IN MUTARE AND MUTASA DISTRICTS OF MANICALAND PROVINCE, ZIMBABWE

By

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A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

I hereby declare that the thesis is my own original work and that, to the best of my knowledge, it contains no material previously published by another person for the award of a degree in any other University, except where acknowledgement has been made in the text.

________________________________________  __________________________

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We, as supervisors confirm that the work reported in this thesis was carried out by the candidate under our supervision. The thesis was examined and we approved it for final submission.

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ABSTRACT

Bionomics of malaria vectors including species composition, resting and biting behaviour and insecticide resistance are important for insecticide-based malaria control and interventions. The lack of data on malaria vector species composition and relative abundance, resting and biting behaviour, as well as insecticide resistance, make the development of target control measures problematic in Mutare and Mutasa Districts. It is imperative to elucidate, characterize, and identify all members of the An. funestus group and the An. gambiae complex to determine their resting and biting behaviour, host-seeking activities, disease relationships, and resistance to insecticides. A longitudinal study was carried out to investigate the bionomics of malaria vector mosquitoes in Mutare and Mutasa Districts. Anopheline larval and adult sampling was conducted from May 2013 to December 2014 using scooping, pyrethrum spray catch, prokopac aspirator, exit window trap, pit shelter, and Centers for Disease Control (CDC) light trap methods. Indoor and outdoor resting mosquitoes were collected in randomly selected houses and pit shelters, respectively. Mosquitoes sampled by light traps were divided into two cohorts. In one cohort, traps were left overnight and mosquitoes collected the following morning, while in the other set, mosquitoes were collected hourly throughout the night. Mosquito samples for insecticide resistance testing were divided into two subsamples. One subsample was used immediately for WHO susceptibility assays and the other batch was allowed to oviposit in the insectary at the National Institute of Health Research, and females from the F1 progeny were used in further susceptibility assays. Mosquitoes were identified using morphological keys and polymerase chain reaction (PCR) techniques. The PCR-based assays showed the presence of four sibling species: Anopheles funestus sensu stricto (90.8%, 267/294) and An. leesoni (5.1%, 15/294) of the An. funestus group and An. arabiensis (41.9%, 13/31) and An. quadriannulatus (48.4%, 15/31) of the An. gambiae complex. Of the two malaria vectors, An. funestus sensu stricto was more abundant (95.4%, 267/280) than An. arabiensis (4.6%, 13/280). Endophilic collections of the An. funestus group and the An. gambiae complex were five times greater than exophilic catches. Nearly 90% endophilic An. funestus populations were collected on sprayable surfaces and the remainder was caught on unsprayable surfaces. Of the sprayable surfaces catches, 56% were collected on the roofs; with 44% on the walls. Of the gravid An. funestus caught, nearly two-thirds (218/330) were collected exiting recently pyrethroid-treated structures, with a 24-hour mortality of less than 10%. The CDC light trap catches were more abundant indoors (68%) than outdoors (32%). Anopheles funestus showed variable indoor and outdoor flight activity rhythms, with two peaks during the night; between 22:00-23:00 hours and 02:00-04:00 hours. Human blood index was 64.3%, with Plasmodium falciparum infection rate of 1.8%. Wild caught females showed resistance to lambda-cyhalothrin (3.3% mortality), deltamethrin (12.9% mortality), etofenprox (9.2% mortality), and bendiocarb (11.7% mortality). F1 An. funestus females showed resistance to deltamethrin (14.5% mortality), lambda-cyhalothrin (6.9 % mortality), etofenprox (8.3% mortality), and bendiocarb (16.8% mortality), but were susceptible to DDT and pirimiphos-methyl (100% mortality). Intensity resistance assay to bendiocarb had 100% mortality, while deltamethrin, lambda-cyhalothrin, and etofenprox had increased knockdown times with mortalities ranging between 66.7 and 92.7% after 24-hour exposures. The present work revealed important information on the behaviour of malaria vector mosquitoes in Mutare and Mutasa Districts, which if not addressed might threaten gains made in malaria control in the study area. It is imperative to change house-spraying insecticide from pyrethroids to organophosphates or DDT (organochlorine), develop an insecticide resistance management
plan, provide extension lances to the house-spraying programme, complement mosquito nets with the use of mosquito repellents and long clothes, and establish a monitoring programme to determine the occurrence and distribution of *An. funestus* populations in Manicaland Province.
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CHAPTER 1

GENERAL INTRODUCTION

1.1 Global burden of malaria

Malaria which is caused by parasites of the genus *Plasmodium* and transmitted by female *Anopheles* mosquitoes is by far one of the most important diseases, despite being preventable and treatable. Globally, 300-500 million cases of malaria occur each year, resulting in over one million deaths (World Health Organization [WHO], 2012). The greatest burden of the disease and deaths is among children under the age of five years who are particularly susceptible to infection, illness and death. Unsurprisingly, more than two thirds (70%) of all malaria deaths occur in this age group (WHO, 2012).

Substantial scale-up of effective interventions between 2000 and 2013 has reduced incidence rates of malaria by 30% globally and by 34% in Africa (WHO, 2014a). During the same period, the global malaria mortality rate decreased by an estimated 47% worldwide, while the decline in Africa was 54%. The proportional reduction in mortality rates in children under the age of five years is estimated to be 53% globally and 58% in the WHO African Region (WHO, 2014a).

The reductions in malaria mortality have contributed immensely to progress towards achieving the target for Millennium Development Goals (MDGs), improving child survival rates around the world by reducing child mortality rate by two thirds between 1990 and 2015 (World Health Organization and United Nations Children’s Fund [WHO and UNICEF], 2015; WHO, 2015a). Between 1990 and 2015, malaria interventions averted about 6.8 million deaths in children under five years of age globally (WHO, 2015a). Plate 1 shows global mortality situation for children under the age of five years.
In the context of morbidity, malaria has devastating socio-economic consequences. For example, malaria has direct expenditure at household level on treatment while governments spend huge amounts of money to procure antimalarial medicines as well as other programmes like vector control and social behaviour change communication (SBCC) (Sachs and Malaney, 2002; Onwujekwe et al., 2008). It is estimated that malaria reduces economic growth rate by about 1.3% (Sachs and Malaney, 2002) and the greatest burden of the disease and deaths primarily lies with the poor, who also have the least access to interventions. As malaria draws global attention, an integrated global approach to its control has been adopted using vector control strategies based on the biology of the mosquito, the epidemiology of the parasite and human behaviour patterns, to prevent continued rise in malaria incidence in the endemic countries (Lengeler, 2004).

1.2 Global distribution of malaria

While malaria transmission occurs in all six WHO Regions (African Region, Region of the Americas, South-East Asia Region, European Region, Eastern and Mediterranean Region and Western Pacific), the disease burden is unevenly distributed and mainly focused in tropical and subtropical regions (WHO, 2007a) where favourable ecological conditions, temperatures, relative humidity and abundance of breeding habitats for malaria vector mosquitoes exist.
Globally, an estimated 3.2 billion people in 97 countries and territories are at risk of malaria infection and developing the disease, and 1.2 billion are at high risk (≥ 1 in 1,000 chance of getting malaria per annum) (WHO, 2014a). From 198 million malaria cases and 584,000 deaths reported globally in 2013, the burden was heaviest in the WHO Africa Region where approximately 90% of all deaths occurred, particularly in children under the age of five years, who accounted for 78% of all deaths (WHO, 2014a). The malaria endemicity varies within regions, ranging from disease risk-free zones to holoendemicity.

Some population groups within the same setting are at considerably higher risk of contracting malaria, and developing severe disease than others. These include infants, children under five years of age, pregnant women and patients with HIV/AIDS, as well as non-immune migrants, mobile populations and travellers. Therefore the National Malaria Control Programmes (NMCPs) need to take special measures to protect these population groups from malaria infection, taking into consideration their specific circumstances.

1.3 Malaria in sub-Saharan Africa

Malaria continues to be one of Africa's biggest killers, with about 90% of all the malaria deaths in the world today occurring in Africa south of the Sahara. The disease intensity is huge in Africa primarily because the majority of infections in the continent are caused by *Plasmodium falciparum*, the most virulent of the five human malaria parasites (WHO, 2002; Hellemond et al., 2009). The majority of people at high risk of contracting the disease live in zones of comparatively stable malaria transmission. In these zones, infection is common and occurs almost all year round with some members of these communities developing immunity against the disease (WHO, 2002).

It is estimated that 36% of the 45 million people in the sub-Saharan African region live in malaria transmission areas (WHO, 2010a) where the disease is rated the second leading cause of morbidity and mortality resulting in about 88% and 90% of the cases and deaths, respectively occurring in the WHO African Region in 2015 (WHO, 2015b). Malaria transmission in most countries in Southern Africa is relatively seasonal (Lengeler, 2004). The distribution of the disease burden largely depends on climate, local ecology, and control interventions that affect
the ability of malaria parasites and the anopheline mosquitoes to coexist long enough to enable transmission (Bruce-Chwatt, 1985). The frequency of transmission or endemicity depends largely on the density and efficiency of the malaria vector mosquitoes.

The problem of the disease in Africa is exacerbated by climate change, poverty and lack of efficient control mechanisms (Tesfaye et al., 2011). In addition, the emerging resistance to antimalarials and insecticides, changes in vector behaviour, human population growth and movement, as well as land use changes and deterioration of public health infrastructures contribute strongly to the spread of the disease (Stefani et al., 2011). To curb malaria transmission in the region, the NMCPs predominantly employ early diagnosis and effective treatment with antimalarial medicines as well as vector control, especially indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) (WHO, 2007a). In addition, Southern African countries have realised that control activities are best achieved if there is cooperation among countries hence the establishment of trans-boundary malaria control initiatives. The initiatives have involved countries such as Zimbabwe, South Africa, Mozambique, Angola, Namibia, Botswana, Swaziland and Zambia, primarily to deliberate on the mechanisms and partnerships necessary for trans-boundary malaria control and elimination in their sub-region.

1.4 Malaria transmission in Zimbabwe

Malaria continues to be a serious public health problem in Zimbabwe and remains one of the most important tropical diseases in the country. Approximately 50% of the population in Zimbabwe live in malaria transmission areas and are at risk of contracting the disease (Midzi et al., 2004). The malaria parasite *P. falciparum*, which is mostly transmitted by the primary vector mosquito *Anopheles arabiensis* Patton, is the predominant species responsible for the most severe and potentially fatal form of the disease in Zimbabwe (Masendu et al., 2005).

While malaria remains one of the most important causes of morbidity and mortality in Zimbabwe, the disease has been gradually declining over the past decade. For the past ten years, Zimbabwe has recorded a steady annual decline in malaria morbidity, from an annual incidence of 55 cases per 1,000 populations in 2003, to 29 cases per 1,000 populations by the end of 2013. Malaria deaths decreased from approximately 3,000 in the early 2000s to about 300 people per
annum in recent years (Global Fund [GF], 2015). A clear reduction in malaria burden was observed in the southern and central parts of Zimbabwe, with Matabeleland South Province recording malaria cases of less than 1 case per 1,000 populations in 2012 (District Health Information System 2 [DHIS 2], unpublished data). Most gains made in malaria control have been associated with widespread implementation of malaria control strategies, especially IRS, LLINs, and early diagnosis and effective treatment.

Although Zimbabwe has experienced a decline in malaria incidence in recent years, the reductions in malaria burden have not been achieved uniformly, with five of the seven districts of Manicaland Province, including Mutare and Mutasa, being among the top 10 high malaria burdened districts in the country (DHIS 2, unpublished data). To maintain gains made in malaria control over the years in Zimbabwe, there is a serious need to strengthen monitoring of malaria vector bionomics (vector species composition and relative abundance, resting and biting behaviour, and insecticide resistance in vector populations), especially in Mutare and Mutasa Districts.

The epidemiology of malaria and intensity is mainly influenced by physio-geographical factors such as low altitudes, high temperatures and rainfall patterns. Malaria transmission in Zimbabwe is seasonal, heterogeneous and mostly unstable, and endemicity ranges from risk-free areas to high malaria transmission zones (Taylor and Mutambu, 1986). The low lying regions to the east and north of the country, with altitudes of below 700 m above sea level have relatively moderate to high malaria transmission, while southern and central areas are low to risk-free zones. The high seasonal malaria transmission patterns predispose people to the risk of malaria outbreaks. The peaks and epidemics are associated with the rainy season and are common between February and April (Taylor and Mutambu, 1986). While all age groups are at risk of malaria, pregnant women, children under the age of five years, the malnourished as well as the immune-compromised have the highest risk of developing severe disease (WHO and UNICEF, 2015). The burden of malaria has serious socio-economic consequences in Zimbabwe. The malaria transmission season generally coincides with the planting and/or harvesting season and brief periods of illness result in loss of production time, leading to a high cost for Zimbabwe.
Anopheles mosquitoes which are the link between the human and malaria parasites have demonstrated a very high level of heterogeneity at macro-geographic scale in Zimbabwe (Taylor and Mutambu, 1986; Masendu et al., 2005), with members of the An. gambiae complex and the An. funestus group being responsible for transmitting malaria in the country. Members of the An. gambiae Giles complex include An. gambiae sensu stricto (hereafter referred to as An. gambiae), An. coluzzii Coetzee and Wilkerson, An. arabiensis, An. bwambiae White, An. melas Theobald, An. merus Dönitz, An. quadriannulatus Theobald, An. amharicus Hunt, Coetzee and Fette and An. comorensis Brunhes, le Goff and Geoffroy. Of these, An. arabiensis is the major human malaria vector in Zimbabwe (Masendu et al., 2005), with An. gambiae a secondary vector. Anopheles gambiae, An. arabiensis, An. merus and An. quadriannulatus have showed some degree of sympatry, although the association between An. arabiensis and An. quadriannulatus is more common (Masendu et al., 2005).

The An. funestus group consists of An. funestus sensu stricto (hereafter referred to as An. funestus), An. vaneedeni, An. leesoni, An. confusus, An. fuscivenosus, An. brucei, An. parensis, An. aruni, An. rivulorum, An. rivulorum-like An. longipalpis type A, An. longipalpis type C, An. funestus-like, An. funestus-like-like (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Spillings et al., 2009 ). Within the An. funestus group, An. funestus is the only member that is implicated as an important vector of malaria in Zimbabwe, and is regarded a secondary vector in the country (Mpofu, 1985; Taylor and Mutambu, 1986; Masendu et al., 2005). Although An. rivulorum., An. parensis and An. leesoni have been incriminated as malaria vectors in some parts of Africa (Gillies and de Meillon, 1968; Wilkes et al., 1996; Cohuet et al., 2003), their role in the transmission of the disease in Zimbabwe is little known.

The importance of malaria vectors in transmitting the disease varies depending on their current bionomics including their abundance, feeding and resting behaviour, as well as vectorial capacity in relation to the deployed vector control interventions (Coluzzi, 1984). The resting and biting behaviour of vector mosquitoes are of fundamental importance to vector control, as these are the traits commonly targeted by IRS and LLINs, respectively. In Zimbabwe, especially in Mutare and Mutasa Districts, vector control is a central and critical component of all malaria control strategies and the use of IRS and LLINs has increased immensely over the past decade as part of an effort towards universal coverage of all populations at risk of contracting the disease.
1.5 The National Malaria Control Programme in Zimbabwe

In order to avert the economic retardation and the suffering caused by malaria, Zimbabwe’s NMCP adopted the use of proven intervention strategies to control the disease. The strategies include early diagnosis and effective treatment with antimalarial medicines, intermittent preventive treatment in pregnancy (IPTp), vector control using IRS and LLINs and SBCC.

Following the current Global Malaria Action Plan (Roll Back Malaria [RBM], 2008), Zimbabwe is going for universal coverage with both preventative and curative interventions in all populations at risk of contracting malaria. The concept is to scale-up preventative measures to full coverage, sustain for extended period, leading to a shift from malaria control to elimination. The initiative is motivated mainly by evidence that malaria morbidity and mortality has been gradually, but steadily declining in many districts in Zimbabwe.

To effectively implement malaria intervention strategies, the Zimbabwe NMCP works with several partners including agencies of the United Nations (UN), non-governmental organizations (NGOs), the private sector and individuals. While working with several stakeholders in malaria prevention and control, the NMCP remains at the centre, coordinating and leading the overall implementation of malaria control programmes in the country. Moreso, to ensure standardisation of all malaria interventions, the NMCP formulates malaria control policies and guidelines including national malaria strategic plan, malaria treatment guidelines, IRS guidelines, and insecticide treated net policy for all nets distributed and used by all malaria stakeholders in Zimbabwe.

1.6 Malaria vector control in Zimbabwe

In Zimbabwe, IRS and LLIN interventions remain the mainstay of malaria control strategies, a situation which is likely to continue due to the remarkably slow development and adoption of alternatives. Several studies have demonstrated the efficacy of IRS and LLINs in curbing malaria transmission in most settings (Lengeler, 2004; Pluess et al., 2010). House-spraying and LLIN strategies are being implemented in 47 districts with moderate to high malaria transmission intensity primarily using dichloro-diphenyl-trichloro-ethane (DDT) and pyrethroids to protect about half of the total population of Zimbabwe. Although house-spraying is strictly indoors,
DDT is commonly used in non-major commercial agricultural zones, whereas pyrethroids are used to protect communities residing in farms which export their produce. Currently, IRS and LLINs depend on four WHO approved classes of insecticide: pyrethroids, organochlorines (OCs), organophosphates (OPs), and carbamates. Of these, pyrethroids account for most IRS coverage globally and are currently used to treat all LLINs (WHO, 2013a).

Mosquito nets played a much lesser role to IRS until the initiation of LLIN campaigns under the universal coverage goal over the past few years. The full employment of the two vector control strategies had no clear rationale to balance LLINs and IRS coverage in Zimbabwe following WHO’s recommendations (WHO, 2014b). Resulting from the full scale inception of LLINs to complement IRS, the two interventions have since been used in combination in the same geographical areas to control malaria transmission in Zimbabwe, protecting people in all districts with moderate to high disease burden. While IRS and LLINs are proven strategies for malaria control (Lengeler, 2004; Pluess et al., 2010), the high dependence on insecticide-based malaria control has the tendency to increase the selection pressure exerted by insecticides on malaria vectors, leading to the emergence of insecticide resistance.

1.7 Malaria in Mutare and Mutasa Districts

Despite much effort on malaria control, the disease remains a major public health problem in Mutare and Mutasa Districts of Manicaland Province, Zimbabwe, with 40 of the 67 administrative wards in the two districts considered high malaria transmission zones. Approximately 95% of cases are caused by *P. falciparum* (Lukwa et al., 2014) and the recent nationwide survey reported that *An. arabiensis* is the primary malaria vector in all regions in Zimbabwe (Masendu et al., 2005). The estimated number of malaria cases in the two districts in 2013 was 257 cases per 1,000 populations (DHIS 2, unpublished data), constituting an incidence rate of 25.7%, which is relatively high for the districts in a country with a long history of malaria prevention and control. The incidence rate is a cause for concern if the two districts have plans to move from malaria control to pre-elimination phase in the near future, as the cases are far above the incidence rate of 0.1% acceptable to move from control to implement malaria elimination activities (WHO, 2007a).
Malaria cases are infrequent from July to October, increasing gradually, with peaks recorded between February and April. In the period from July to October, low temperatures in July to August inhibit parasite development in mosquitoes while dry conditions in September and October are unfavourable for mosquito survival and breeding success, especially members of the *An. gambiae* complex. The onset of rains in November up to April and high temperatures result in more favourable conditions for mosquitoes, leading to high malaria transmission in the two districts. Mutare and Mutasa Districts commonly experience sporadic malaria epidemics around March/April which at times are devastating, despite their control within a few weeks with guidance from the Zimbabwe NMCP.

To prevent malaria transmission and sporadic outbreaks in Mutare and Mutasa Districts, Zimbabwe’s NMCP uses early diagnosis and effective treatment with antimalarials, IPTp, vector control and SBCC. Vector control, especially deployment of IRS and LLINs is considered key for the prevention of malaria transmission in the two districts. Larval source management using biolarvicides, particularly *Bacillus thuringiensis israelensis* has been employed as a supplementary measure in winter when the breeding sites are limited, especially in Mutare District which has less and more manageable larval habitats than Mutasa District.

Mutare and Mutasa Districts are included in Zimbabwe’s long history of vector control against members of the *An. gambiae* complex dating back to the late 1940s with IRS using DDT and pyrethroids as the main pillar of malaria interventions for over 30 years (Mabaso et al., 2004). Following Zimbabwe’s NMCP policy and guidelines on the rational use of insecticides for vector control, Mutare and Mutasa Districts have used only pyrethroids as the two districts export agricultural produce. Of the pyrethroid products, deltamethrin and lambda-cyhalothrin have been used interchangeably. However, the selection of the two insecticides has not been based on an insecticide resistance management plan, instead, it was principally based on the allocated budget for insecticide procurement.

In Mutare and Mutasa Districts, LLINs have been distributed recently to complement IRS, with the first mass net distribution campaign not occurring until 2008 (Lukwa et al., 2014). This was in 30 high malaria burdened districts. In recent years, house-spraying and LLIN interventions have been scaled-up in Mutare and Mutasa Districts following the national campaign for
universal access to malaria interventions. This has resulted in substantial reductions in disease burden (346 to 257 cases per 1,000 populations from 2009 to 2013 respectively) (DHIS 2, unpublished data) and human exposure to malaria vector mosquitoes. In 2010, economic and political support from the Government and international partnerships has led to the distribution of 87,377 and 80,000 LLINs in Mutare and Mutasa Districts respectively. Meanwhile, IRS coverage was 95% and 99% in 2010 compared to 98% and 92% in 2009 in Mutare and Mutasa Districts, respectively (DHIS 2, unpublished data).

Both IRS and LLINs have been shown to be effective in reducing malaria transmission when applied independently in any setting (Lengeler, 2004; Kim et al., 2012), hence their wide application in Mutare and Mutasa Districts. In an effort to speed up the control and ultimate elimination of malaria in future, IRS in combination with LLINs has been deployed in the same households in Mutare and Mutasa Districts despite poor evidence as to whether the combined use of the two interventions in the same geographical setting is more effective in reducing the incidence of malaria than using either intervention independently (Okumu and Moore, 2011; Kleinschmidt et al., 2009; Pluess et al., 2010; Yakob et al, 2011). It is poorly understood how the two insecticide-based interventions interact to improve malaria control in any setting (Deressa et al., 2016). Besides, the effectiveness of IRS and LLINs in reducing malaria transmission strongly depends on the bionomics of malaria vector mosquitoes, especially resting and biting behaviours, as well as insecticide resistance in mosquito populations. Efficacy of IRS against malaria vectors largely depends on whether mosquitoes prefer to rest indoors (endophilic behaviour) or outdoors (exophilic behaviour). The resting behaviour varies among species and is affected by insecticide irritancy and repellency. Exophilic behaviour has developed in certain mosquito populations exposed to prolonged house-spraying programmes, leading to outdoor malaria transmission. Behavioural resistance in malaria vectors in some countries worldwide has arisen in response to prolonged house-spraying with DDT or pyrethroids, leading to strong stimulation in mosquitoes to take off and fly without having taken up the lethal dose of insecticide (Pates and Curtis, 2005).

The work of Gerold (1977) in Tanga region of Tanzania demonstrated insecticide irritancy in An. gambiae where high proportions of fully ergoged mosquitoes were observed leaving the sprayed structures. A study in Gokwe and Binga Districts in Zimbabwe showed partial exophilic traits in
the principal vector *An. arabiensis* (Masendu, 1996). The observed outdoor resting behaviour has the tendency to maintain malaria transmission in an area where IRS is a major intervention, despite high coverage. Predominantly endophilic mosquito populations may include varieties that exhibit exophilic tendencies, a phenomenon which was considered the reason for the failure of a WHO house spraying programme to control malaria in the district of Garki, Nigeria in the late 1960s to the mid-1970s (Molineaux and Gramiccia, 1980).

To control malaria vectors which bite indoors, Mutare and Mutasa Districts use LLINs with the aim of achieving the national campaign goal of universal access to LLINs for all populations at risk of contracting malaria. Effectiveness of LLINs against malaria vectors fundamentally depends on whether mosquitoes prefer to bite humans (anthropophilic). Of those vector species that bite humans, efficacy of LLINs as intervention tools also relies on whether vectors are nocturnal and bite indoors (endophagic behaviour) or outdoors (exophagic behaviour). To gain maximum protection by LLINs, the peak period of mosquito feeding must coincide with times when most people would be in bed under mosquito nets.

Long-lasting insecticidal nets have had a remarkable result on the reduction in incidence of malaria in several Asian countries and on mortalities in Africa (Lengeler, 2004). It has been feared that the effectiveness of LLINs may be compromised if the biting times and/or venues change in an area where mosquito net use is the major strategy to curb malaria transmission. The work by Charlwood and Graves (1987) in Papua New Guinea set the scene for recognising a marked shift towards biting at sunset by *An. farauti* when mosquito nets were introduced in the country. More recently, a study in the Solomon Islands showed that, due to insecticide pressure, *An. farauti* changed its behaviour to biting early in the evening outdoors avoiding insecticide exposure indoors (Bugoro *et al*., 2011).

Although several studies have shown the efficacy of IRS and LLINs in reducing malaria incidence in almost all settings (Lengeler, 2004; Pluess *et al*., 2010), outdoor malaria transmission and the emergence of insecticide resistance pose a serious challenge to the milestones gained over the years in malaria control and elimination. Resistance in malaria vector populations has developed to every insecticide class, including microbial drugs and insect growth regulators (Brogdon and McAllister, 1998; WHO, 2012).
Even though insecticides have been used for a very long period in Zimbabwe, there are very few instances when resistance has been recorded (Munhenga et al., 2008). Early reports of insecticide resistance in *An. arabiensis* appeared in the 1980s in Chiredzi District and showed benzene hexachloride resistance (BHC) (Green, 1982). Masendu et al. (2005) and Munhenga et al. (2008) reported resistance in *An. arabiensis* to DDT and permethrin in Gokwe District. No further insecticide resistance was documented until recently when pyrethroid and carbamate resistance was reported in *An. funestus* in Mandeya ward of Mutasa District (Choi et al., 2014). The same study showed 100% susceptibility to DDT and OPs. Insecticide resistance is expected to directly and greatly affect the re-emergence of vector-borne diseases (Krogstad, 1996), and where resistance has not contributed to disease resurgence, it is expected to threaten disease control in any setting (WHO, 1992).

Although changes in the malaria vector bionomics, especially shifts from indoor to outdoor resting and biting behaviour, as well as insecticide resistance in mosquito populations are perceived as serious threats to the future of malaria control, current information on the behaviours of vector mosquitoes is patchy in Mutare and Mutasa Districts, and not much is known about their severity in the two areas. The responses of malaria vector mosquitoes to prolonged use of pyrethroid-based IRS and LLINs in the two districts are little understood. It is therefore imperative that a careful study to obtain current and comprehensive information on malaria vector species composition and relative abundance, resting and biting behaviours, as well as insecticide resistance in malaria vector populations be conducted in Mutare and Mutasa Districts. Provision of such information is fundamental to malaria control and elimination.

### 1.8 Research problem and justification

Despite being preventable and treatable, malaria continues to have a devastating effect on people’s health and livelihoods around Mutare and Mutasa Districts in Zimbabwe. Recent efforts to control malaria have realised reduction in number of cases. The malaria incidence rates declined from 37.2% in 2003 to 25.7% in 2013 in both districts (DHIS 2, unpublished data). Even though the incidence of malaria declined over the past decade, the disease burden remains comparatively high in the districts despite a long history of vector control (Mabaso et al., 2004).
In Mutasa District, there was a resurgence of malaria in 2007, after more than five years of successful control. In 1998, Mutasa District reported 67,978 cases of malaria, which were reduced to 19,883 in 2002, and in 2007, cases rose to 75,884 (Moss et al., 2012). These cases of malaria in Mutasa District demonstrate the dramatic resurgence of the disease if control interventions are not maintained or cease to function owing to the changes in the bionomics of the vector mosquitoes. To sustain the milestones achieved in malaria control in recent years in Mutare and Mutasa Districts, it is imperative to understand changes in malaria vector biology and status of insecticide resistance. The information is important for the development of sustainable and effective malaria control interventions to sustain the gains as well as to prevent resurgence once a control level is achieved.

Vector control is a core component of malaria prevention and control in both districts. This involves the use of IRS and LLINs as the main malaria prevention interventions. The two strategies have been deployed in combination in the same geographical areas for the past few years. While the two strategies have shown good results in various settings (Lengeler, 2004), they have their bottle necks. The main problems with house-spraying and use of LLINs are the shifts in vector populations to rest and bite outdoors, as well as development of insecticide resistance.

The behaviour of various malaria vectors in relation to vector control interventions has been broadly studied (Pates and Curtis, 2005). In some areas, *An. arabiensis* is known to bite extensively outdoors in the early evening (Govella et al., 2010) while most people are outdoors and awake, and some studies have suggested that vectors may adopt such behaviours in response to prolonged and high coverage of IRS or LLINs (Braimah et al., 2003). More recently, observations showed altered feeding rhythms of mosquitoes resulting in a shift of biting venues and times from occurring indoors at night when people are asleep to outdoors in the evenings and mornings following increased use of IRS and LLINs in the Solomon Islands (Bugoro et al., 2011), Equatorial Guinea (Reddy et al., 2011), and Tanzania (Russell et al., 2011). Masendu (1996) reported partial exophilic behaviour in *An. arabiensis* in Gokwe District, Zimbabwe — an area commonly sprayed using DDT. These observations are important for Zimbabwe’s vector control programme in Mutare and Mutasa Districts as the two areas may have the same
challenges of malaria vector mosquitoes resting and biting outdoors whilst continuing to deploy IRS and LLIN tools indoors, rendering the two malaria control strategies less effective.

Shifts in the behaviours of vector mosquitoes to rest and bite outdoors when IRS and LLINs are deployed indoors may allow persistence of malaria transmission, despite high coverage and use of the two intervention strategies by the majority of the people at risk in Mutare and Mutasa Districts. The impact of the induced outdoor resting and biting behaviours to malaria control is exacerbated by emergence of insecticide resistance in vector populations. Over the years, some cases of emergence of insecticide resistance in vector populations, especially An. arabiensis has been reported in various parts of Zimbabwe (Green, 1982; Masendu et al., 2005; Munhenga et al., 2008; Choi et al., 2014).

The heavy reliance on insecticide-based vector control and the increased outdoor malaria transmission, as well as the emergence of insecticide resistance in major malaria vector populations put national, provincial and district efforts at risk. Even with the current scaling-up of insecticide-based vector control in Mutare and Mutasa Districts, little will be achieved in malaria control, unless there is consistent monitoring of the emergence of new vector species, their resting and biting behaviours as well as insecticide resistance in the mosquito populations. With no current information on malaria vector bionomics (vector distribution, resting and biting behaviours, and resistance status to insecticide), the milestones achieved over the years could be reversed in Mutare and Mutasa Districts.

Despite the number of studies on vector distribution, resting and biting behaviours, as well as resistance status to insecticides in Zimbabwe, those that looked at vector species composition, their resting and biting behaviours, and susceptibility status to insecticide in Mutare and Mutasa Districts are not available. Most of the researchers on malaria and vectors in Zimbabwe have concentrated along the Zambezi Valley, the Lowveld, Gokwe and Chiredzi Districts (Green, 1982; Masendu et al., 2005; Masendu, 1996; Dandalo, 2007; Munhenga et al., 2008). Information on mosquito species composition and relative abundance, resting and biting traits, as well as vector resistance to insecticides is important for malaria control and elimination in the two districts.
The present work outlined comprehensive malaria vector species composition and relative abundance, resting and biting behaviours, and the insecticide resistance status of vector mosquito populations collected in Mutare and Mutasa Districts. The information is essential for future entomological monitoring and the overall improvement of malaria vector control strategies.

1.9 Objectives

1.9.1 Overall objective

The aim of the study was to investigate bionomics (relative abundance, resting, feeding, insecticide resistance) of malaria vector mosquitoes in Mutare and Mutasa Districts.

1.9.2 Specific objectives

Specifically, the objectives of the study were to:

i. determine malaria vector species and their relative abundance in Mutare and Mutasa Districts;

ii. determine resting behaviours of mosquitoes in Mutare and Mutasa Districts;

iii. assess biting behaviours of mosquitoes in Mutare and Mutasa Districts; and

iv. evaluate the susceptibility levels of malaria vector mosquitoes to all classes of insecticides recommended for malaria vector control in Mutare and Mutasa Districts.

1.10 Hypotheses

i. *Anopheles arabiensis* is the principal malaria vector in Mutare and Mutasa Districts.

ii. Malaria vectors in Mutare and Mutasa Districts are endophilic and endophagic.

iii. Malaria vector mosquitoes in Mutare and Mutasa Districts are susceptible to all four classes of insecticides approved by the WHO for use in IRS: pyrethroids, carbamates, organophosphates and organochlorines.
CHAPTER 2
LITERATURE REVIEW

2.1 Overview of Mosquitoes

Mosquitoes belong to the order Diptera, family Culicidae (Service, 1996). There are about 3,200 species and subspecies of mosquitoes belonging to 37 genera, with most pests and vector species belonging to the genera Anopheles, Culex, Aedes, Psorophora, Haemogogus and Sabethes (Service, 1996). Of these, Anopheles, Culex and Aedes mosquitoes are the most important group because of their role in transmitting different parasitic diseases to various communities. Mosquitoes which transmit malaria belong to the genus Anopheles, whereas species in other genera including Culex and Aedes can also serve as vectors of disease agents, but not malaria (Service, 1996). In addition to malaria transmission, some Anopheles species are also vectors of other parasitic diseases including filariasis and various arboviruses, but malaria is the most worrisome.

2.1.1 Morphological description

Mosquitoes belong to the phylum Arthropoda. Arthropods include spiders, beetles, ticks, butterflies and mosquitoes (WHO, 2003) and information for distinguishing mosquitoes from other arthropods is important for the control of mosquito-borne diseases. Adult mosquitoes can be recognized by their thin, elongated and bilaterally symmetrical body which reaches a length of approximately 16 mm and covered with an exoskeleton. The body is divided into head, thorax and abdomen, with three pairs of long legs and a pair of thin transparent wings (WHO, 2003). The whole body is segmented, with most frequently yellowish brown or grey colour. The abdomen extends anteriorly from the thorax and consists of 10 segments. Each leg is provided with a pair of claws. The wings are covered with scales, which are grouped together to form clusters. The head is provided with one pair of antenna and palp, and a proboscis. The antennae are long and consist of 15 segments (WHO, 2003). Adult female often has a long proboscis ending in a bristle that is modified for piercing the skin and sucking blood meals. The proboscis of a male mosquito is blunt hence males are not able to pierce skins and suck blood (Rueda, 2008).
Anophelines can morphologically be distinguished from culicines by their resting positions and the length of proboscis. Anophelines rest at an angle between 50° to 90° to the surface, whereas culicines rest more or less parallel to the surface (WHO, 2003). Additionally, in female anophelines, palps are as long as proboscis, while in female culicines, palps are shorter than proboscis. In male anophelines, palps are as long as proboscis and clubbed at tip, and in male culicines, palps are longer than proboscis, with tapered tips (WHO, 2003). Mosquito larvae are distinguished from other aquatic insects by the absence of legs, the presence of head with mouth brushes and antennae and either a pair of respiratory openings in Anophelinae or an elongated siphon in Culicinae mosquitoes extending from the posterior of the abdomen (Rueda, 2008).

2.1.3 Geographical distribution

Mosquitoes constitute a large and abundant group that has an almost worldwide geographical distribution, occurring throughout the tropics and temperate regions, and stretching outside the Arctic Circle. However, mosquitoes are absent only from a few islands and Antarctica (Rueda, 2008). The biodiversity of mosquitoes is distinct, with most genera having worldwide occurrence, while a few have limited distribution. *Anopheles*, *Culex* and *Aedes* mosquitoes have at least one species found in all five regions (Africa, America, Asia, Australia and Europe) of the world (WHO, 2003). In all five regions, subfamily Culicinae (81-88%) has greater number of species than subfamily Anophelinae (Rueda, 2008). A nationwide survey in Zimbabwe showed distribution of *Anopheles* and *Culex* mosquitoes in various parts of the country and in most cases the two genera are found in sympatry (Masendu et al., 2005).

2.1.4 General biology and ecology

Mosquitoes have successive different stages of growth and metamorphosis in their life cycle, and these are egg, larva, pupa and adult. The female deposits more than 100 eggs at a time either singly (*Anopheles* and *Aedes*) or in clusters (*Culex*) in open waters (Rueda, 2008). In some ecological settings, eggs attach themselves on the surface of aquatic plants. Aquatic habitats vary within mosquito species, as habitat preferences contribute to the occupation of different ecological niches (Rueda, 2008). Mosquitoes can thrive in a variety of habitats with fresh water, brackish water, salt water or any water body which is clear or turbid or polluted (WHO, 2003). The first three stages in the development of mosquitoes (egg, larva and pupa) occur primarily in
The length of these three stages ranges between 5-14 days, depending on the species and environmental conditions. There are some exceptions, especially in cold or dry regions. In such environments, mosquitoes enter a state of diapause (delayed development), usually lasting several months (WHO, 2003).

The larvae develop into first, second, third, fourth instars and pupae. The pupae or tumblers appear after the fourth instars and do not feed. The pupae live for 1-3 days before becoming adults (WHO, 2003). Blood-sucking mosquitoes have a life cycle lasting from one week to a few months, depending on the type, sex and environmental conditions (WHO, 2003). Adult female mosquitoes bite humans and animals as a blood meal is required to produce viable eggs. Males feed primarily on plant juices and nectars. Some species prefer human host (anthropophilic), while others feed on animals including birds (zoophilic) (WHO, 2003). There are some mosquito species that readily feed on fish exposed to air, reptiles, amphibians, and insect larvae (Harwood and James, 1979).

### 2.2 Malaria vector mosquitoes

There are approximately 400 different species of *Anopheles* mosquitoes globally, with only about 60 being vectors of malaria under natural settings and of these, about 30 sibling species are considered to be of major public health importance (Bruce-Chwatt, 1985). The most important malaria vectors in Africa south of the Sahara are members of the *An. gambiae* complex and the *An. funestus* group (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Service, 1996; Coetzee et al., 2000). Of these species, *An. funestus* (a member of the *An. funestus* group), *An. gambiae* Giles, *An. coluzzii* and *An. arabiensis* (of the *An. gambiae* complex) are known to be major malaria vectors and are responsible for more than 95% of all malaria cases in Africa (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee et al., 2013). In Zimbabwe, *An. arabiensis* is the most widely distributed (Plate 2), and is responsible for most of the malaria transmission, with *An. gambiae*, *An. coluzzii* and *An. funestus* being secondary vectors (Masendu et al., 2005; Coetzee et al., 2013).
2.2.1 Anopheles gambiae sensu lato (complex)

2.2.1.1 Morphological description

Before 1962, An. gambiae complex was initially considered to be a single species primarily because members of the complex are morphologically identical (Coetzee et al., 2000; Sinka. 2013). However, there were many reports which showed genetically and behaviourally distinct sibling species within the complex that differed noticeably in their ability to transmit the diseases (Coluzzi, 1978). Currently, the complex is considered to include about nine morphologically identical sibling species and these are An. gambiae, An. coluzzii, An. arabiensis, An. bwambae, An. melas, An. merus, An. quadriannulatus, An. amharicus and An. comorensis (White, 1985, Hunt et al., 1998, Coetzee et al., 2013).

Plate 2: Distribution of An. funestus and some members of the An. gambiae complex in Zimbabwe (Masendu et al., 2005)
2.2.1.2 Geographical distribution

The distribution of malaria vectors, especially those belonging to species complexes that contain non-vector species, is important for strategic planning for malaria prevention and control (Coetzee et al., 2000). Although members of the *An. gambiae* complex cannot be reliably distinguished morphologically, they differ in their geographical occurrence. Members of the *An. gambiae* complex have been reported from most countries of Africa and its adjacent islands, including Madagascar, as well as Saudi Arabia and Yemen (Coetzee *et al*., 2000). In sub-Saharan Africa, members of the *An. gambiae* complex are the most widespread and potent malaria vector species (Sinka, 2013). Several studies on malaria vector distribution in Zimbabwe reported sympatric occurrence of members of the *An. gambiae* complex in various parts of the country (Mpofu, 1985; Taylor and Mutambu, 1986; Masendu *et al*., 2005). Although *An. gambiae* and *An. arabiensis* occur widely in sympatry over several countries in Africa, *An. gambiae* is absent from all areas to the north-east of Uganda and the Kenya Highlands, while in West Africa its range extends into the Sahel in Mauritius. In addition, there is a general absence of *An. arabiensis* from the forest belt and more humid areas of West Africa (Coetzee *et al*., 2000). In contrast, work by Coluzzi *et al*. (1979) showed that *An. arabiensis* established itself in towns in southern Nigeria within the forest belt, but could not extend into surrounding countryside. These observations would seem to rule out simple climate-based explanations for the differences in distribution of mosquito species.

The identification to sibling species of field-collected mosquitoes is of practical importance. Green (1982) reported a typical case of confusing a mixture of two or more sibling species when treated as a single population. When WHO assays for dieldrin resistance were carried out on *An. gambiae* complex collected from three localities in Zimbabwe, confusing results were obtained with 98% survival rate at one site, 0.8% at the second site and 43% on the third site. When the specimens were identified cytogenetically, most of the dead mosquitoes were shown to be *An. quadriannulatus* which is not a vector of *Plasmodium*. Some of the survivors were identified as *An. arabiensis* and were therefore resistant to dieldrin and a spraying campaign with BHC (which exhibited cross resistance with dieldrin) by health authorities would have had no effect on disease control (Gillies and Coetzee, 1987).
2.2.1.3 Biology and ecology

Members of the *An. gambiae* complex differ in their ecology and each species has different breeding habitats preferences, including freshwater species (*An. gambiae, An. coluzzii, An. arabiensis, An. quadriannulatus, An. amharicus and An. comorensis*), saltwater species (*An. melas and An. merus*), and mineral water species (*An. bwambae*) (Gillies and Coetzee, 1987). Paterson (1964) published the results of a study at Chirundu, Zambia, showing the occurrence in combination of sympatry of freshwater species, *An. gambiae, An. arabiensis* and *An. quadriannulatus*. In Zimbabwe, Masendu et al. (2005) reported that freshwater species, *An. gambiae, An. arabiensis* and *An. quadriannulatus*, and saltwater species, *An. merus* co-existed in nature. *Anopheles gambiae, An. coluzzii* and *An. arabiensis* are the most important malaria vectors of the complex and are highly anthropophilic but, when alternative mammalian hosts are available, *An. arabiensis* shows a much greater tendency to bite animals, clearly demonstrating its opportunistic feeding traits (Gillies and Coetzee, 1987). *Anopheles quadriannulatus* and *An. amharicus* are mostly zoophilic and are not considered to be involved in malaria transmission. Meanwhile little is known about the biology of *An. comorensis* (Lanzaro and Lee, 2013) and its actual existence as a taxon is yet to be confirmed (Coetzee et al., 2013).

2.2.2 *Anopheles gambiae sensu stricto* and *An. coluzzii*

2.2.2.1 Geographical distribution

*Anopheles gambiae* is widely distributed in nearly all African countries south of the Sahara and is the world’s most efficient malaria vector (Gillies and De Meillon, 1968; Bruce-Chwatt, 1985). The species is almost universal in tropical Africa except at high altitudes and deserts; often limited in dense forest areas. To the northern part of Africa, its occurrence ranges from the extreme south of Mauritania to the southern borders of the Sahara through the northern Sudan to the Red Sea coast of Arabia near Jedda (Gillies and De Meillon, 1968). In southern Africa, it occurs in several countries including Botswana, Mozambique, Zambia and Zimbabwe, but is absent from the central plateau, which includes the southern Transvaal, Orange Free State, Cape Province and Lesotho (Gillies and De Meillon, 1968; Coetzee et al., 2000; Masendu et al., 2005). In the Indian Ocean it is present in Madagascar, Reunion, Mauritius and the Comoros (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). The distribution of *An. coluzzii*
extends from the northern Senegal in the west to east-central Africa and south to coastal Angola, with one specimen identified from Zambezi Valley in Zimbabwe (Coetzee et al., 2013).

2.2.2.2 Biology and ecology

Anopheles gambiae prefers to breed in a variety of water bodies, especially in open and shallow habitats, with adequate sunlight. The preferred habitats include hoof prints, pools, puddles, and burrow pits, as well as rice and yam fields (Gillies and De Meillon, 1968). Work by Minakawa (1999) on An. gambiae habitats showed that the species prefer open than shaded habitats for oviposition mostly because larval predation is less in temporary than permanent water bodies, and open water bodies are associated with plenty of natural larval food. Moreso, warmer temperatures encountered in small and open habitats during the day shorten aquatic developmental stages, while also reducing mortality associated with dehydration.

The water bodies in open sunlit pools used for breeding may be clear or muddy, while the survival of the larvae in mud is reduced to only a few hours (Thomson, 1945; Gillies and De Meillon, 1968). However, pools with suspended colloidal matter, such as those often found in brick-pits, seem to be particularly favourable (Gillies and De Meillon, 1968). Although the influence of pH to mosquito aquatic life was extensively studied, there is little information on the importance of physical and chemical factors in An. gambiae breeding-sites (Gillies and De Meillon, 1968). Work by Namuchimba (2007) in Gokwe District, demonstrated that mosquito larval density was generally not affected by pH, conductivity, total dissolved solids, dissolved oxygen, calcium, nitrates and chlorides.

While An. gambiae prefers cool temperatures, the species can also survive in comparatively high temperatures and reports have shown its presence in dry climates, with seasonal variation in densities, being more common in wet than dry periods (Gillies and Coetzee, 1987). However, the density tends to fluctuate following the season and rainfall pattern, resulting in high densities during the rainy season and becoming low when it is dry. In Zimbabwe, which experiences one rainfall season, the densities rise considerably soon after the first rains in November, with the peak in March/April (Taylor and Mutambu, 1986). In equatorial zones with two rainy seasons, An. gambiae has two peaks (Gillies, 1964). In contrast, work by Heisch (1947) at Isiolo in
northern Kenya observed one peak which followed the short rains (October to December) rather than the long rains (March to June).

*Anopheles gambiae* is extremely anthropophilic with greater preference to feed on human blood (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). In some few instances, *An. gambiae* has been observed feeding on domestic animals; even in such situations, the human blood index was high, exceeding 90% (Gillies and De Meillon, 1968). The preferences by *An. gambiae* to feed on human blood maintains the life cycle of human parasites, contributing enormously to its role as the most efficient malaria vector globally. In most areas, the feeding by *An. gambiae* takes place indoors, primarily connected to its late feeding behaviour (Gillies and Coetzee, 1987). The predominantly indoor feeding habit of *An. gambiae* makes the use of LLINs more effective in the prevention of malaria transmission in localities where this species is the primary vector.

Upon taking enough blood meals, most *An. gambiae* mosquitoes prefer to rest indoors, making house spraying highly effective in prevention of malaria transmission. Across the country in Zimbabwe, studies by Mahon *et al.* (1976) and Mpofu (1985) on malaria vector species composition showed distribution and relative abundance of *An. gambiae* in several sites. Twenty years later, similar studies by Masendu *et al.* (2005) observed *An. gambiae* only in Kanyemba area in the Zambezi Valley, Mashonaland Central Province, Zimbabwe. The scarcity of this species was principally attributed to decades of nationwide house spraying using mostly DDT.

*Anopheles coluzzii* is associated with longer lasting breeding habitats mainly resulting from human activity. In the savannah, the habitats include rice fields, reservoirs and drainage ditches. In forest areas they appear to be urban pools, sometimes polluted (Coetzee *et al.*, 2013).

### 2.2.3 *Anopheles arabiensis*

#### 2.2.3.1 Geographical distribution

*Anopheles arabiensis* is the most widely distributed member of the complex found in most of the countries in Africa south of the Sahara (Morlais *et al.*, 2005) and is a major malaria vector in Zimbabwe (Mpofu, 1985; Taylor and Mutambu, 1986; Masendu *et al.*, 2005). The distribution of *An. arabiensis* is concentrated in the lower rainfall regions, which represent the drier savannah
areas (Coetzee et al., 2000), and this is in agreement with the known habitats of this species (Coluzzi et al., 1979). Where *An. arabiensis* occurs in equatorial rainforest regions, it is usually associated with history of extensive land deforestation, as was reported in Benin and Nigeria (Coluzzi et al., 1979). In southern Africa, the species is distributed in several countries including Angola, Botswana, Mozambique, Namibia, Malawi, Zambia and Zimbabwe, among others (Gillies and Coetzee, 1987; Coetzee et al., 2000; Masendu et al., 2005).

### 2.2.3.2 Biology and ecology

The species has similar breeding habits to *An. gambiae*, except that its distribution and relative abundance is primarily dependent upon geographical conditions and climatic factors (Lindsay et al., 1998). The species shows a greater tolerance to hot and dry environments where it tends to survive better than in wet conditions (Edillo et al., 2004). Its tolerance to high temperatures and survival in dry conditions is linked to extensive biting in dry situations. For *An. arabiensis* mosquitoes to survive in hot and dry conditions, with the females laying eggs in moist surfaces in place of water and even though there is a delay in the hatching of eggs, the mosquito population is maintained (Lindsay et al., 1998). This habit probably clarifies the observation of *An. arabiensis* in Matabeleland South Province (Masendu et al., 2005), one of the driest regions in Zimbabwe.

While *An. arabiensis* mosquitoes are highly anthropophilic, the vectors tend to change the feeding preferences when alternative mammalian hosts are available, leading to greater tendency to feed on animals (Gillies and Coetzee, 1987). Since most domestic animals are kept in shelters outside homes at night, this gives *An. arabiensis* a good opportunity to bite domestic animals and rest outdoors. The opportunistic habit by this vector mosquito to feed on both humans and animals renders use of LLINs less effective for preventing malaria transmission. Feeding on animals by some *An. arabiensis* mosquitoes is a good opportunity for Zimbabwe’s NMCP to implement zooprophylaxis (diverting mosquitoes from biting humans to animals). As was observed in studies in Tanzania, *An. arabiensis* was diverted from feeding on humans to cattle, offering protection against mosquito bites and consequently reducing malaria incidence (Mahande et al., 2007).
The exophilic behaviour of *An. arabiensis* mosquitoes has the tendency of reducing the effectiveness of house spraying on its population in comparison to *An. gambiae* (Service *et al.*, 1978). Therefore, in regions where *An. arabiensis* is the principal vector, implementation of house-spraying as a major malaria control measure may not control malaria to the level of ceasing to be of public health importance (White, 1974). *Anopheles arabiensis* is the principal malaria vector in Zimbabwe (Masendu *et al.*, 2005) and its outdoor resting tendencies that were demonstrated in Gokwe and Binga Districts (Masendu, 1996), might pose a serious challenge to Zimbabwe’s house-spraying programme for malaria control.

### 2.2.4 *Anopheles quadriannulatus, An. amharicus* and *An. comorensis*

#### 2.2.4.1 Geographical distribution

*Anopheles quadriannulatus* occurs widely in Zimbabwe, South Africa, Mozambique and Swaziland, whereas *An. amharicus* is found in Ethiopia (Hunt *et al.*, 1998; Coetzee *et al.*, 2000; Coetzee *et al.*, 2013). *Anopheles comorensis* was described and named by Brunhes *et al.* (1979) based on the morphology of a single specimen from the Indian Ocean Island of Comoros in the Mozambique Channel (Lanzaro and Lee, 2013; Coetzee *et al.*, 2013). Until such time as adequate genetic confirmation of species distinctness is established, the specific status of *An. comorensis* and its association with *An. gambiae* complex remains questionable (Coetzee *et al.*, 2013). *Anopheles quadriannulatus* is found in sympatry with malaria vectors including *An. gambiae, An. arabiensis* and *An. funestus* in most parts in southern Africa (Coetzee *et al.*, 2000; Coetzee *et al.*, 2004; Masendu *et al.*, 2005).

#### 2.2.4.2 Biology and ecology

*Anopheles quadriannulatus* was previously classified into two sibling species A and B. Currently, *An. quadriannulatus* is the former *An. quadriannulatus* species A, and *An. amharicus* is the former *An. quadriannulatus* species B (Lanzaro and Lee, 2013). *Anopheles quadriannulatus* and *An. amharicus* are primarily zoophilic and are not considered to be involved in the transmission of malaria. The zoophilic habits of *An. quadriannulatus* have been confirmed in Ethiopia by White (1980) and Boreham (1973). Meanwhile, the biology of *An. comorensis* is not clearly understood (Brunhes *et al.*, 1997; Lanzaro and Lee, 2013; Coetzee *et al.*, 2013).
2.2.5 *Anopheles merus*

2.2.5.1 Geographical distribution

*Anopheles merus* is patchily distributed in Africa, mainly confining itself along the East African coast including limited inland localities with saltwater bodies (Gillies and Coetzee, 1987; Sharp, 1983). The work by Coetzee *et al.* (1993) in Mozambique and South Africa, and Masendu *et al.* (2005) in Zimbabwe, demonstrated inland presence of *An. merus* mosquitoes.

2.2.5.2 Biology and ecology

The species is commonly found breeding in salty lagoons, ponds, swamps and pools which are diluted by rainfall or seepage from the ground (Thomson, 1945). Laboratory tests have demonstrated that survival of larvae was high in freshwater and in concentrations of up to 60% sea-water but declined sharply at higher concentration salinity (Gillies and Coetzee, 1987).

Depending on the environment, the majority of adults can be caught resting indoors by day. A study in Tanzania showed that *An. merus* species that enters houses to feed, 35 to 40% would leave the houses the same night to rest and complete gonotrophic cycle outside (Thomson, 1951a). In some areas, adults are largely exophilic and can be collected resting outside on the bases of mangroves and mango trees, as well as in termite holes and other similarly shaded hollows (Gillies and De Meillon, 1968). While three key studies reported the occurrence of *An. merus* in various localities in Zimbabwe (Mahon, 1976; Masendu, 1996; Masendu *et al.*, 2005), its resting and biting behaviour could not be investigated.

While the most preferred hosts by *An. merus* are domestic animals, in the absence of the preferred blood meal, the species bites humans both indoors and outdoors. It appears that its importance as a malaria vector is limited, though in some localities where it is abundant, the huge number may partly make up for a lowered vectorial capacity. A study by Thomson (1951b) showed 0.8% positive for sporozoites in the *An. merus* specimens collected in Dar-Salam, Tanzania. Although the infection rate is low in *An. merus*, it demonstrates its role as a malaria vector in localised settings. Masendu *et al.*, (2005) showed that *An. merus* was implicated in malaria transmission at Masakadza area in Gokwe District.
2.2.6 *Anopheles melas*

2.2.6.1 Geographical distribution

*Anopheles melas* has a limited occurrence in Africa. It is present in the West African coast from the Senegal River to the mouth of the Congo, and southwards to Lobito in Angola (Gillies and De Meillon, 1968). Little is known about its occurrence in Zimbabwe (Masendu et al., 2005).

2.2.6.2 Biology and ecology

*Anopheles melas* is also a salt-water member of the *An. gambiae* complex. The species is broadly distributed along the West African coastline with saltwater breeding sites, being capable of thriving in water of very high salinity (Gillies and Coetzee, 1987). Aquatic stages have been found in patches of salt grass in tidal swamps and pools, and mangrove swamps (Gillies and De Meillon, 1968). Even though *An. melas* is a coastal species, it occurs in saline conditions in areas inland from the open sea (Coetzee et al., 2000). On perusal of available literature, it appears there is no document evidence for the occurrence of this species in Southern Africa.

Several studies by Thomson (1948) and Gelfand (1955) have shown that even in the presence of domestic animals, *An. melas* would prefer human host for its blood meal. It is more endophagic than exophagic and the proportion of those that immediately exit houses after taking blood meal indoor is much greater than in *An. gambiae* (Gillies and De Meillon, 1968). In Lagos, Nigeria, Thomson (1948) found that at least 43% left the houses the same night after taking blood meals, while about 75% of those that had remained, exited the structures at dusk the following evening. As a result, the house-resting population was less than half the number that fed indoors. In areas where this species is a malaria vector of public health concern, using IRS as a major tool to control *An. melas* might not give the desired results (Gillies and Coetzee, 1987).

*Anopheles melas* is an important malaria vector in various parts along the West African coast and in certain places it can be the principal vector during most months of the year except during wet season when *An. gambiae* is abundant and becomes the major vector (Gillies and Coetzee, 1987). Malaria infection in *An. melas* is generally low. Bryan (1983) found a sporozoite rate of 0.32% in about 5,000 dissected *An. melas* collected at Brefet, The Gambia.
2.2.7 *Anopheles bwambae*

2.2.7.1 Geographical distribution

The geographical distribution of *An. bwambae* is enormously localised and only known from the small area of the Rift Valley, west of Ruwenzori, where appropriate ecological conditions exist (Gillies and Coetzee, 1987).

2.2.7.2 Biology and ecology

*Anopheles bwambae* is the only member in the *An. gambiae* complex which prefers to breed in mineral water bodies. Abundant breeding occurs in pools which originate from hot mineral springs in the Semliki forest in Bwamba area, Uganda (Gillies and Coetzee, 1987). Studies have shown that larvae appear to be greatly dependent on the minerals in the water bodies, and attempts to rear them in tap or distilled water have not been successful (Davidson and White, 1972), contrary to the tolerance shown by salt-water species of the *An. gambiae* complex to fresh water in the laboratory (Gillies and Coetzee, 1987).

The species exhibits anthropophilic traits, with more indoor biting and outdoor resting habits. White (1973) indicated that most females that take blood meals and rest indoors would leave the structures at dusk the following evening, leading to abundant collection of males and females resting outdoors, especially on tree trunks and buttresses in the forest. In addition, it was demonstrated that domestic pigs were attractive to *An. bwambae* for blood meal, although less so than human sources. *Anopheles bwambae* has been implicated in malaria transmission and work by White (1973) reported sporozoite rate of 0.7% in specimens collected indoors from Bwamba, Uganda.

2.2.8 *Anopheles funestus* group

2.2.8.1 Morphological description

*Anopheles funestus* group is one of the most important populations of mosquitoes owing to the exclusive involvement in the transmission of human malaria by one or more members of the group. The term “*Anopheles funestus* group” was first devised in its strict sense by Gillies and De Meillon (1987) to designate members into sub-group of species morphologically close to *An. funestus* (Table 1). Currently, substantial evidence shows that a group of species belong to the
taxon *An. funestus*, with different morphological, behavioural and epidemiological characteristics (Dia *et al.*, 2013). These species show minor or no morphological differences at adult stage and identification to sibling species can be currently achieved through the use of polymerase chain reaction (PCR) assays. It is now accepted that *An. funestus* mosquitoes belong to a group composed of five subgroups of which three subgroups comprise about 14 sibling species which occur in Afrotropical regions (Table 1) (Dia *et al.*, 2013).

Table 1: Members of the *Anopheles funestus* group in Afrotropical regions and their roles in malaria transmission (Dia *et al.*, 2013) with minor adjustments

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Species</th>
<th>Geographical distribution</th>
<th>Host preference</th>
<th>Vector role</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. funestus</em></td>
<td><em>An. funestus</em> s.s.</td>
<td>Continental</td>
<td>anthropophilic</td>
<td>major</td>
</tr>
<tr>
<td></td>
<td><em>An. funestus</em>-like</td>
<td>Local</td>
<td>Unknown</td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td><em>An. funestus</em>-like-like</td>
<td>Local</td>
<td>Unknown</td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td><em>An. aruni</em></td>
<td>Local</td>
<td>Unknown</td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td><em>An. confusus</em></td>
<td>Regional</td>
<td>Zoophilic</td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td><em>An. parensis</em></td>
<td>Regional</td>
<td>Unknown</td>
<td>minor</td>
</tr>
<tr>
<td></td>
<td><em>An. vaneedeni</em></td>
<td>Local</td>
<td>Unknown</td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td><em>An. longipalpis</em> type C</td>
<td>Local</td>
<td>Zoophilic</td>
<td>unknown</td>
</tr>
<tr>
<td><em>An. minimus</em></td>
<td><em>An. leesoni</em></td>
<td>Continental</td>
<td>Zoophilic</td>
<td>minor</td>
</tr>
<tr>
<td></td>
<td><em>An. longipalpis</em> type A</td>
<td>Local</td>
<td>Zoophilic</td>
<td>unknown</td>
</tr>
<tr>
<td><em>An. rivulorum</em></td>
<td><em>An. rivulorum</em></td>
<td>Continental</td>
<td>Zoophilic</td>
<td>minor</td>
</tr>
<tr>
<td></td>
<td><em>An. rivulorum</em>-like</td>
<td>Local</td>
<td>Unknown</td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td><em>An. brucei</em></td>
<td>Local</td>
<td>Unknown</td>
<td>unknown</td>
</tr>
<tr>
<td></td>
<td><em>An. fuscivenosus</em></td>
<td>Local</td>
<td>Unknown</td>
<td>unknown</td>
</tr>
</tbody>
</table>

### 2.2.8.2 Geographical distribution

Of the members of the *An. funestus* group, *An. funestus*, *An. leesoni* and *An. rivulorum* are the most widely distributed species (Plate 3). They occur throughout sub-Saharan Africa (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Being predominantly a savannah mosquito species (Ayala *et al.*, 2009), the major malaria vector in this group, *An. funestus* occurs in many other localities, including high altitude zones, 900 m in Madagascar (Andrianaivolambo *et al.*, 2010), 1,440 m in Central Africa (Tchuinkam *et al.*, 2010), and up to 2,000 m in Kenya (Okara *et
Anopheles funestus is patchy or completely absent along the coast (Ayala et al., 2009). This malaria vector disappeared from several parts of Africa following harsh climatic conditions and/or vector control programmes (Mouchet et al., 1996). Recently, several countries have reported the re-emergence of this species once control measures had been stopped or emergence of insecticide resistance or suitable environmental conditions re-appeared (Fontenille and Rakotoarivony, 1998; Hargreaves et al., 2000; Dia et al., 2010). Anopheles funestus was a major vector of malaria in South Africa in the early 1950s (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). The vector species was eradicated from the country following house-spraying using DDT ((Hargreaves et al., 2000). Since then it has been reported only once from an unsprayed area and was quickly eliminated again with use of IRS (De Meillon et al., 1977). More than 50 years later, the country experienced malaria outbreak following discontinuation of use of DDT for house-spraying. Anopheles funestus sibling species was implicated in the epidemic signifying its resurgence in South Africa (Hargreaves et al., 2000; Coetzee, 2006).
The other members of the *An. funestus* group are locally distributed. *Anopheles parensis*, *An. confusus* and *An. aruni* are localised in East Africa (Kamau et al., 2003; Muturi et al., 2009), *An. rivulorum*-like and *An. brucei* in West and Central Africa (Hackett et al., 2000; Cohuet et al., 2003), and *An. vaneedeni*, *An. parensis*, *An. fuscivenosus*, *An. funestus*-like, *funestus*-like-like, as well as *An. longipalpis* types A and C in southern Africa (Gillies and Coetzee, 1987; Spillings et al., 2009; Koekemoer et al., 2009).

### 2.2.8.3 Biology and ecology

Of the members of the *An. funestus* group, *An. funestus* is considered one of the most proficient malaria vectors globally (Gillies and De Meillon, 1978). Its role goes beyond that of a secondary vector, exceeding *An. gambiae* in several parts of Africa (Coetzee and Fontenille, 2004). It appears the species was largely neglected in comparison to members of the *An. gambiae* complex, threatening the current regional campaign for malaria elimination, and the lack of attention on this vector is mostly due to the difficulty of adapting this species to standard insectary conditions (Dia et al., 2013).

*Anopheles funestus* commonly breeds in natural or artificial semi-permanent and permanent water bodies with floating or emerging vegetation, with more preference to emerging vegetation (Gillies and De Meillon, 1968). Natural breeding sites include the edges of swamps, weedy and grassy river/stream banks, as well as ditches and ponds. The presence of vegetation is of great importance for mosquito breeding since aquatic stages strive best in shaded breeding sites and can hardly survive in water bodies in direct sunlight. Artificial habitats are mostly dams, wells and irrigation channels and the development of aquatic stages is adversely affected by salinity, high temperatures and at times persistent heavy rains (Gillies and De Meillon, 1968).

The breeding habits of other members of the *An. funestus* group are little understood. However, several studies on the *An. funestus* group suggested that the breeding habits of the majority of other members of the group are similar to those of *An. funestus*, and at times occur in sympatry (Gillies and De Meillon, 1968; Abou-Nasr, 1970; Gillies and Coetzee, 1987; Minakawa et al., 2012).
All malaria transmission studies have shown that *An. funestus* is the major malaria vector in the group, with *P. falciparum* infection rates exceeding those of other malaria vectors (Temu et al., 2007). Early records of its involvement in malaria transmission gave infection rates as high as 22% in South Africa (Gillies and De Meillon, 1968). More recently, in Tanzania, 11% sporozoite rate was recorded (Shiff et al., 1995), 5% in southern Mozambique (Aranda et al., 2005), and exceptionally 50% in Burkina Faso (Costantini et al., 1999). In Zimbabwe, *P. falciparum* parasite rates up to 11% in *An. funestus* were recorded (Evans and Leeson, 1935).

*Anopheles funestus* is the most anthropophilic, endophagic and endophilic member of the group. Overall, *An. funestus* exhibits relatively consistent human host preferences and indoor resting habits in different environments (Awolola et al., 2005; Dabire et al., 2007). In savannah regions where breeding habitats are dependent upon rainfall patterns, *An. funestus* extends malaria transmission during dry season when the densities of *An. gambiae* and/or *An. arabiensis* decline (Fontenille et al., 1997; Dia et al., 2003). However, due to its strong endophilic and endophagic behaviour, *An. funestus* is most vulnerable to control with house-spraying and LLINs, respectively (Pates and Curtis, 2005). Mpofu (1985) observed occurrence of *An. funestus* in several parts of Zimbabwe, and 20 years later, the species occurred only in one locality (Masendu et al., 2005), following scaling-up of house-spraying using mostly DDT.

Other members of the group are primarily zoophilic, although they occasionally take human blood (Gillies and De Meillon, 1968), with most species preferring to rest indoors (Smith, 1961; Adugna and Petros, 1996; Hargreaves et al., 2000; Kent et al., 2006). Positive *P. falciparum* infected specimens of *An. rivulorum*, *An. leesoni* and *An. parensis* were observed in Tanzania (Wilkes et al., 1996; Temu et al., 2007), suggesting a secondary roles of these mosquito species in malaria transmission. *Plasmodium falciparum* infected *An. parensis* specimens were observed in South Africa (Mouatcho et al., 2007). The role of *An. vaneedeni* and *An. longipalpis* in malaria transmission is little known (Adugna and Petros, 1996; Kent et al., 2006; Mouatcho et al., 2007).
2.3 Species identification

Sibling species identification of members of the An. gambiae complex and the An. funestus group are essential for vector control programmes in Africa (Sharp et al., 1989). Speciation is of particular importance in sub-Saharan Africa where members of the An. gambiae complex and the An. funestus group occur in sympatry. Entomological monitoring usually loses its importance if recorded without isolating sibling species from the complex (Sharp et al., 1989), and the development of reliable tools to identify members of the complex adds value to the roles played by entomology in malaria prevention and control (Dia et al., 2013).

Commonly used identification methods include polytene chromosome technique, electrophoresis, and more recently polymerase chain reaction (PCR). The use of PCR increased widely following the public release of the genome sequencing of members of the An. gambiae complex (Scott et al., 1993). This event had a large effect on a better understanding of the complexity of malaria vectors. In addition, the publication of the An. gambiae genome sequencing was a great opportunity to a rapid development of new genetic tools, from molecular markers to transgenic mosquitoes (Besansky, 2008). Currently, protocols exist to identify members of the An. gambiae complex (Scott et al., 1993) and of the An. funestus group (Koekemoer et al., 2002; Spillings et al., 2009) using PCR assays.

2.4 Bionomics of Anopheles vectors

Bionomics is that part of biology (often called autecology) which deals with the relationships of a given species and its environment (WHO, 1975). A study on bionomics of mosquito species includes their biology, aquatic stages, species composition, abundance, insecticide resistance, as well as resting and biting behaviour. Current information on the bionomics of malaria vector mosquitoes is essential as the success of any malaria control programme using vector control strategies primarily depends on targeting one or more behaviour of vector mosquitoes.

Changes in the environment may have a direct or indirect effect on the behaviour of vectors, consequently reducing the effectiveness of vector control tools to control malaria transmission (WHO, 1982). To strengthen vector control programmes, it is of paramount importance to regularly update information on vector species composition, resting and biting behaviour, as well
as insecticide resistance status in malaria vectors. Insecticide resistance in major malaria vector mosquitoes is believed to have immensely contributed to the failure of global effort to eradicate malaria in some countries in the mid-1950s (Pates and Curtis, 2005).

2.5 Malaria parasites

Human malaria is caused by the infections of the protozoan parasites *Plasmodium falciparum*, *P. ovale*, *P. malariae* and *P. vivax*, and more recently *P. knowlesi*, restricted to the family Plasmodiidae, within the order Coccidiida (Gillies, 1993; Hellemond et al., 2009). Of these, *P. falciparum* is the most virulent and dangerous form of malaria (Kitua et al., 1996), with the highest rates of complications and mortality. The remaining four are less common and less dangerous *Plasmodium* species. *Plasmodium vivax* is not common in Africa because the Duffy blood antigens (the erythrocyte molecules to which its merozoites bind) are rare in the African populations (May et al., 1999).

Plate 4 shows the life cycle of *P. falciparum* in the human and in the mosquito. The first sexual developmental stage of the parasites involves gametocytes being ingested along with asexual stages by the mosquito in the blood meal of an infected human. Fertilization will result into a diploid zygote or ookinete. The zygote moves to the gut, depending on the parasite species, the ookinete ruptures in 8-16 days, releasing sporozoites that make their way to the salivary glands of the mosquito (Foster and Walker, 2002). The sporozoites are passed to the next human when the mosquito gets the subsequent blood meal. The sporozoites get to the liver where they reproduce asexually to form merozoites which enter the red blood cells upon rupturing of liver cells. The merozoites in the red blood cells also undergo asexually reproduction to produce more merozoites and the cycle is repeated (Foster and Walker, 2002). The merozoites form male and female gametocytes which are taken up by the blood-sucking female *Anopheles* species to repeat the cycle in the body of the mosquito (Foster and Walker, 2002).

The severity of the disease is dependent on the parasite species and general health and degree of immunity of the infected person (WHO, 1995). Mixed sporozoite infections involving two or more *Plasmodium* species are common. Asymptomatic and non-fatal cases of malaria may last more than five months depending on the immune system of the infected person (Foster and
Walker, 2002). Common signs and symptoms of simple malaria include headache, nausea, diarrhoea, vomiting, muscle pain, weakness, chills, fever and sweating, whereas severe cases are associated with breathing difficulties, severe anaemia, confusion, coma and death.

Plate 4: Life cycle of the most important malaria parasite *P. falciparum* in both the human and the mosquito ([http://www.parasitesinhumans.org/plasmodium-falciparum-malaria.html](http://www.parasitesinhumans.org/plasmodium-falciparum-malaria.html))

### 2.6 Global malaria eradication efforts: successes and failures (1955-1978)

With the success of DDT in interrupting malaria transmission, the WHO submitted at the World Health Assembly in 1955 a proposal for the eradication of malaria worldwide (WHO, 2015a). Eradication efforts began and focused on house-spraying with residual insecticides, antimalarial medicines and surveillance, and were carried out in four successive steps: preparation, attack, consolidation, and maintenance (Tanner and de Savigny, 2008). Successes included elimination in most European nations with temperate climates and seasonal malaria transmission. Some countries such as India and Sri Lanka had sharp reductions in the number of cases, followed by increases to substantial levels after efforts ceased (Tanner and de Savigny, 2008). Other nations had negligible progress including Indonesia, Afghanistan, Haiti and Nicaragua, while sub-
Saharan Africa was deemed “not ready” (Martin et al., 2004) and excluded completely from the global campaign, which therefore ignored the territories that recorded 90% of the malaria cases and deaths in the world at that time (Malagón, 2008).

The emergence of parasite resistance to antimalarial medicines, mosquito resistance to insecticides, wars and massive population movements, as well as difficulties in obtaining sustained funding from donor countries, and lack of community participation made the long-term maintenance of the effort untenable (Tanner and de Savigny, 2008). Completion of the eradication campaign was eventually abandoned. Following the aborted Global Malaria Eradication campaign, malaria received little international attention over the subsequent years until recently (Tanner and Savigny, 2008). After the launch of the RBM programme in 1998, most countries with endemic malaria, especially in Africa, made substantial progress in their malaria control interventions (RBM, 1998). To reduce malaria transmission to a level where it is no longer a public health problem is the goal of malaria prevention and control. Recent increases in resources, political will, and commitment have led again to discussion of the possibility of malaria elimination and, ultimately, eradication, using mainly vector control strategies, IPTp and effective treatment (Tanner and de Savigny, 2008).

### 2.7 History of vector control

Prior to the Second World War, malaria vector control was largely by environmental management through drainage and landfills to eliminate the larval mosquito habitats, biological control through the use of larvivorous fish in ponds, as well as larviciding with oil and Paris green (Mabaso et al., 2004). The control measures were mostly dependent upon accurate information to distinctly differentiate vector mosquito preferences of breeding habitats (WHO, 2013b). There is evidence that environmental management had a significant effect in reducing the disease burden (Takken et al., 1990; Keiser et al., 2005), even though malaria elimination was not yet on the global agenda. While environmental management strategies were effective, especially in Europe, malaria transmission continued to be a serious concern on a global scale (Najera, 2000).
The introduction of DDT and other organochlorine insecticides during the early 1940s saw a dramatic shift from environmental management to insecticide-based vector control (Najera, 2000). House-spraying with insecticides reduced the average longevity of vector mosquitoes to below the age at which they become infectious (MacDonald, 1956), extensively reducing the burden of malaria. Following wide scale use of insecticide-based vector control, malaria was even eliminated from a number of countries, especially in Europe. However, increased insecticide and antimalarial resistance, as well as inadequate funding resulted in a failure of the global campaign to eliminate malaria in other countries (WHO, 2012). The successes of DDT in the elimination of malaria in some countries was an opportunity for private sectors to develop other classes of insecticides, the most recent and common class being pyrethroids, developed in the 1980s (WHO, 2012). Pyrethroids are currently the most common insecticides used for vector control.

Vector control is a core strategy in malaria control. The strategy principally involves the use of house-spraying with insecticides or LLINs (WHO, 2014a). Larval source management can be used as a supplementary strategy under specific conditions (WHO, 2013b). World Health Organization (2015a) currently recommends universal coverage with effective vector control interventions of all populations at risk of contracting malaria. Together, IRS and LLINs account for almost 60% of global investment in malaria control (RBM, 2008).

The number of LLINs delivered has increased intensely in recent years, rising from 6 million in 2004 to 145 million in 2010 in sub-Saharan Africa (WHO, 2013c). Approximately 300 million LLINs were delivered to African countries between 2008 and 2010. Long-lasting insecticidal nets have played an important role in the remarkable success of reducing malaria burden over the past decade (WHO, 2014a). Similarly, the number of people protected from malaria by IRS in the WHO African Region increased from 10 million in 2005 to 78 million in 2010. In total, 185 million people were protected by IRS in 2010, representing 6% of the global population at risk (WHO, 2011).
2.7.1 House-spraying and mosquito nets for malaria control in Zimbabwe: past and present

In Zimbabwe, IRS was started as a pilot study in 1949 using DDT and then BHC (Alves and Blair, 1953, 1955), and is currently the mainstay of malaria vector control in the country. In 1986, deltamethrin, a synthetic pyrethroid, was evaluated in Zimbabwe in experimental huts and the residual effect was found acceptable for malaria vector control (Taylor et al., 1981). In the same year, micro-encapsulated deltamethrin was tried under field conditions and recommended for widespread house-spraying to curb malaria transmission in Zimbabwe (Taylor et al. 1986). Later, in 1990, lambda-cyhalothrin, another pyrethroid was tested in a small community in Zimbabwe and the residual activity was comparable to deltamethrin, hence was also recommended for countrywide use in malaria vector control, leading to the interchangeable use of the two insecticides in the country (Lukwa et al., 2012).

In recent years, IRS in Zimbabwe adopted the recommendations by the WHO (2015c) of universal coverage to populations at risk, through striving to achieve a minimum spray and population coverage of 80%. The spray coverage for Zimbabwe varied considerably over the years, ranging from 64% in 2001 to 92% in 2014 (DHIS 2, unpublished data), and the disparity may be attributed to inadequate funding, especially in the early 2000s.

To complement house-spraying, the Zimbabwe NMCP introduced untreated mosquito nets in the early 1990s up to 2003/4 when they were replaced with insecticide-treated nets (ITNs). Presently, the country has shifted from ITNs to LLINs in compliance with a global call by World Health Organization (2007b) to all National Malaria Control Programmes and their partners involved in malaria prevention using ITNs to purchase only LLINs. Traditionally, mosquito nets played a much lesser role to IRS in Zimbabwe, until the initiation of LLIN campaigns under the universal coverage goal in recent years. In an effort to accelerate the control and ultimate elimination of malaria, IRS in combination with LLINs have been deployed in the same districts and geographical areas in Zimbabwe, with the aim of controlling indoor resting mosquitoes, as well as those species that feed indoors and rest outdoors, usually missed when house-spraying is used independently. Today, it appears there are no policies or guidelines in Zimbabwe regarding
whether to combine IRS and LLINs in the same geographical area or independently deploy each strategy.

2.7.2 Resting behaviour in relation to house-spraying

The effectiveness of house-spraying with insecticides against malaria vectors depends on the preferences by the mosquitoes to rest indoors (endophilic traits). The resting habits vary among species and are affected by insecticidal irritancy or repellency (Pates and Curtis, 2005). Outdoor resting (exophilic) behaviour has developed in certain mosquito populations exposed to prolonged IRS, leading to the reduced effect of house-spraying with insecticides to curb malaria transmission. Indoor residual spraying with insecticides sufficiently reduces the longevity of *Anopheles* mosquitoes which rest indoors to greatly reduce the possibility of malaria transmission (MacDonald, 1957). House-spraying was the principal strategy by which malaria was eradicated in the temperate zones, as well as the reduction in malaria incidence in India (Pates and Curtis, 2005).

There are now emerging serious problems of behavioural resistance in vectors in some countries in response to prolonged house-spraying programmes, especially using DDT or pyrethroids, adversely affecting the success of the control programme (Pates and Curtis, 2005). Work by Smith and Webley (1968) demonstrated the irritant effect of DDT in verandah trap huts where mosquitoes inside sprayed structures were irritated and exited huts sooner than those in unsprayed huts. In Greece, spraying using DDT led to exophilic habits in the originally endophilic vector, *An. sacharovi*. It was thought that the survival of this species in sprayed areas was as a result of a high level of repellence and irritability and physiological resistance which might have led to the more zoophilic habits of this vector species (de Zulueta, 1959). Similarly, malaria transmission by *An. sundaicus* in southern Java was not interrupted by house-spraying using DDT because of increased outdoor resting behaviour (Sundavaraman, 1958).

In India, where malaria control heavily relies on house-spraying, a major resurgence of malaria occurred in the 1970s, and the problem was associated partly to the existence of outdoor resting populations of the major malaria vector *An. culicifacies* (Pates and Curtis, 2005). Li et al. (1983) showed that residual house-spraying launched in Hainan Island, China, eliminated the malaria
vector, but recent malaria epidemics have since implicated *An. minimus* mosquitoes. Entomological investigations have shown a complete shift in the behaviour of *An. minimus* from endophilic to exophilic.

In Tanzania, Gerold (1977) reported that *An. gambiae* changed its behaviour to rest outdoors soon after feeding indoors, while in Burkina Faso, similar traits were observed with 94% exit rate of *An. funestus* and *An. gambiae* from pyrethroid-treated huts (Darriet, 1991). In Binga and Gokwe Districts, Zimbabwe, Masendu (1996) reported that the principal vector *An. arabiensis* was partially exophilic, suggesting that it might not be fully amenable to control by indoor application of residual insecticides, posing a challenge to malaria control programme. Similarly, Dandalo (2007) demonstrated the strong exophilic behaviour of *An. gambiae* complex in Gokwe District; an area where house-spraying with DDT is the major strategy to reduce malaria transmission.

### 2.7.3 Biting behaviour in relation to use of mosquito nets

Long-lasting insecticidal nets are one of the leading vector control strategies which can be very effective in reducing malaria transmission (Over *et al.*, 2004; O’Meara *et al.*, 2010). The efficacies of this control measure rely greatly on the behaviour of malaria vectors, and are more effective against mosquito species which bite indoors (endophagic behaviour), late in the night (nocturnal) during hours which coincide with the times when most people are in bed (Pates and Curtis, 2005). These characteristics are typically observed for the principal malaria vector mosquitoes in Africa south of the Sahara, and are substantially contributing to the successes of the Malaria Control Programmes in the region (Gillies and Coetzee 1987; Pates and Curtis 2005, O’Meara *et al.*, 2010). In some parts of the world, the feeding traits of vectors vary, with some primary vectors preferring to bite outdoors. In the southeast Pacific, the feeding behaviour of vectors is much more variable, with some species exhibiting a tendency to bite early in the night, outdoors (exophagic behaviour) (Taylor, 1975; Charlwood *et al.*, 1985). Such habits could reduce the efficacy of LLINs strategy which relies strongly on vectors biting indoors.

Some changes in vector behavior from indoor to outdoor biting and resting habits have been attributed to use of IRS and LLINs as was reported for the main vector *A. gambiae* in Bioko
Island, Equatorial Guinea (Reddy et al., 2011). However, blood-fed An. gambiae mosquitoes have been collected exiting houses in window traps, indicating a degree of indoor feeding and outdoor resting behaviour (Molina et al., 1996). In various settings, An. gambiae exhibits high level of anthropophagy (Killeen et al., 2001). Despite this distinct host preference, An. gambiae will bite animals in the absence of available human hosts (Sousa et al., 2001). Such shifts in behaviours may also apply to LLINs to which the prolonged implementation of the strategy may induce changes in vector behaviour from endophagic to exophagic tendencies.

Some insecticides which are used to treat LLINs have excito-repellent effect that may induce selection pressure for outdoor biting habits, where treated nets strictly kill endophagic mosquitoes (White, 1974; Pates and Curtis, 2005). It may therefore be that outdoor venues could become important locations for host-seeking Anopheles mosquitoes, rendering indoor-based LLINs less effective. Additionally, the effectiveness of LLINs is seriously threatened by shifts in biting behaviour, from nocturnal towards twilight, with mosquitoes actively host-searching during early morning and/or evening when many users of the tool would not be under nets (Takken, 2002; Gatton et al., 2013). Work by Russell et al. (2011) in Tanzania observed increased outdoor feeding of malaria vectors following prolonged exposure to insecticide-treated nets. Such a shift could occur simply if mosquitoes are unsuccessful in finding blood meal during their normal active host-searching period, and mosquitoes would “learn” (Chilaka et al., 2012) and always repeat behaviour that resulted in a successful blood meal.

Despite extensive coverage and prolonged use of ITNs in Uganda, malaria morbidity remained high (Kabbale et al., 2013). The high malaria cases were strongly attributed to change in the biting pattern of a greater proportion of the malaria vectors to feeding earlier in the evening and/or morning and feeding outdoors when many people are not in bed, reducing the effectiveness of bed nets (Maxwell et al., 1998), leading to the increase in malaria transmission rates. In Temotu Province, Solomon Islands, Bugoro et al. (2011) showed that, due to insecticide pressure, An. farauti changed its behaviour to biting early in the evening outdoors thus avoiding insecticide exposure indoors.

Despite the number of studies on malaria vectors in Zimbabwe, those that looked at biting behaviours are limited. Work by Dandalo (2007) in Gokwe District showed that the peak indoor
biting rhythm of *An. gambiae* complex occurred at 22:00 hours. Mosquito outdoor biting behaviour was not evaluated in this study.

### 2.8 Insecticides used for vector control

There are a variety of insecticides used for malaria vector control to attack either the aquatic stages or the adult mosquitoes. Larvicides are chemical or biological products designed to be applied directly to breeding sites to control mosquito larvae, while insecticides which are known to be neurotoxic are used in house-spraying or fogging to control adult mosquitoes (WHO, 2003). Synergists are not toxic to the mosquitoes themselves, but they enhance the efficacy of insecticides. There are different classes of insecticides used in public health, agriculture and at household level, and the classification is according to their chemical composition, port of entry into the body of an insect, method of application and the stage of the life cycle of the insect that they are used (Bruce-Chwatt, 1985).

Four classes of insecticide are recommended by the WHO for use against adult mosquitoes in public health programmes includes pyrethroids, organochlorines, organophosphates and carbamates (WHO, 2012). New insecticides or insecticide formulations which appear potentially useful for public health usually undergo a process of independent testing. This is coordinated by the World Health Organization Pesticide Evaluation Scheme (WHOPES). The process ascertains the insecticides’ effectiveness, safety for humans and the environment, and the methods and conditions of their use. World Health Organization Pesticide Evaluation Scheme works in close collaboration with national disease and pest control programmes, national pest registration authorities, legislation and regulation authorities, several international and regional organizations and institutions in pesticides management, as well as research institutions and industries (WHO, 2012).

#### 2.8.1 Pyrethroids

Synthetic pyrethroids are a major class of neurotoxic insecticides, and are still the mainstay of vector control programmes, especially IRS and LLINs. Currently, it is the only class of insecticides recommended for treating LLINs (WHO, 2012). The group has several compounds including deltamethrin, lambda-cyhalothrin, permethrin, cypermethrin and alpha-cypermethrin.
Zimbabwe’s IRS programme has been using deltamethrin and lambda-cyhalothrin interchangeably over a decade in several districts with high altitudes including Mutare and Mutasa Districts in Manicaland Province.

Pyrethroids are synthesised derivatives of naturally-occurring pyrethrins, which are taken from extracts of dried chrysanthemum flowers. Full-scale commercial production of pyrethrins began in the mid-19th century, the major ingredients being pyrethrin one and pyrethrin two which are still in use today in house-spraying (Davies et al., 2007). The first stable compounds, with high insecticidal efficacy, low mammalian toxicity, and limited soil persistence were achieved in the period between 1968 and 1974 (Elliott, 1980). The first of these compounds was permethrin, followed by cypermethrin and deltamethrin, with the latter being the most used insecticide ever known (Davies et al., 2007).

Pyrethroids affect both the peripheral and central nervous systems by interacting with voltage-gated-sodium channels of mosquitoes (Davies et al., 2007). They interfere with ionic conductivity of nerve membranes by prolonging the sodium current. This initially stimulates nerve cells to produce repetitive discharges, eventually causing paralysis of the vector mosquito (Davies et al., 2007).

2.8.2 Organochlorines

The major and commonly known compound in the organochlorine class is DDT. The other members of this family which have been used in malaria control are lindane and dieldrin. Both insecticides are no longer recommended: lindane because of widespread of resistance in major malaria vector mosquitoes and dieldrin because of its high toxicity to humans (Najera and Zaim, 2001).

DDT has been one of the most successful insecticides ever developed (Davies et al., 2007). Even though DDT was developed back in the 19th century, only around 1939 when the Swiss Chemist Paul Muller discovered that it was effective in killing various types of insects was it then used for controlling insect pests (Davies et al., 2007). The discovery of the insecticidal properties of DDT was probably the most important development in the history of pest control. DDT appeared to be the ideal insecticide, with high toxicity to most pests but relatively harmless to humans, as
well as long residual effect on sprayed surfaces. The first full-scale reported use of DDT was during the Second World War. An outbreak of typhus fever in Naples, Italy in December 1943 was brought under control within two weeks by mass treatment of more than 2.5 million people with 10% DDT powder, which controlled the body lice responsible for transmitting the disease (Davies et al., 2007). This created an opportunity for malaria control in the 1950s as DDT was found to be comparatively cheap, safe, and effective against the targeted pests, particularly house-spraying to control malaria vectors, as well as in agriculture against crop pests (Davies et al., 2007).

House-spraying against malaria vectors in Europe was successful, leading to a decision for global malaria eradication. Currently, the continued use of DDT for vector control is conditionally approved under an international agreement — the Stockholm Convention on Persistent Organic Pollutants — aimed at protect both human health and the environment (World Health Organization and United Nations Environmental Programme [WHO and UNEP], 2008). The restriction is partly due to their unacceptably long persistence in the environment and organisms owing to its insolubility in water and its relatively high solubility in fats. These two properties result in long-term accumulation of DDT in the environment and in fatty tissues of non-targeted organisms. Despite these challenges, the Convention has given an exemption for public health use of DDT, mainly because of the absence of equally effective and efficient alternatives (WHO and UNEP, 2008).

DDT affects mostly the peripheral nervous system by triggering neurons to fire spontaneously causing muscles to twitch, with resulting tremors throughout the body and appendages. After some hours, DDT exposure leads to either convulsion (random uncontrolled contraction of the muscles) or paralysis (complete loss of muscle control) and consequent death of the insect. In comparison with pyrethroids, DDT is a slow acting insecticide (Davies et al., 2007).

2.8.3 Organophosphates

Organophosphates were produced in the early 1950s, and the compounds in this group include malathion, pirimiphos-methyl and fenitrothion. The class differs from organochlorines in being much less persistent in the environment and non-target organisms (lasting only a few days), and
is generally much more poisonous to both mosquitoes and humans (Davies et al., 2007). Use of organophosphates in vector control, especially the more toxic compounds has decreased in recent years (WHO, 2006a). In Zimbabwe, organophosphate compounds have not been the insecticide class of choice for vector control over the past years, till recently when their use increased slightly.

The organophosphorus compounds have a non-specific mode of action in mosquito populations. They combine well with the enzyme acetylcholinesterase (AChE), resulting in the inhibition of hydrolysis of the acetylcholine produced at the nerve ending to transmit nerve impulses across the synapses (Insecticide Resistance Action Committee [IRAC], 2010). When mosquitoes are exposed to organophosphorus compounds, acetylcholine accumulates in the synapses, resulting in constant nervous system stimulation leading to paralysis and death. As the organophosphates bind powerfully to both mosquitoes and human AChE, some organophosphates tend to be more toxic to humans and require weekly AChE monitoring of spray operators to assess any possible toxicity during the IRS campaign (IRAC, 2010).

2.8.4 Carbamates

Carbamates were developed in the 1960s, and their persistence and toxicity is between organochlorines and organophosphates (Ware, 1989). Compounds in the group commonly used against malaria vectors include bendiocarb and propoxur. Most members of this group have been widely used to control agricultural pests. Perusal of literature in Zimbabwe showed that carbamates have not been used for vector control on a large scale. The action of carbamates is on the nervous system by the accumulation of acetylcholine at the nerve synapses. Rather than inhibiting AChE, they act as competitors with acetylcholine for the enzyme’s surface (IRAC, 2010).

2.9 Insecticide resistance in malaria vectors

Currently, the most important challenges to malaria control and elimination are the absence of vaccine, parasite resistance to antimalarial medicines and vector resistance to insecticides (WHO, 2007a). Insecticide resistance is a reduction in sensitivity of an insect population as reflected by repeated failure of an insecticide to achieve the expected level of control when used
according to recommendations (IRAC, 2010). Resistance has been observed in more than 500 insect species globally, among which more than 50 species are malaria vectors (Hemingway and Ranson, 2000). Resistance is a heritable characteristic that relies on the genetics of living organisms. Resistance results from the selection of a genetic modification in one or several genes occurring by migration and/or mutation. In general, when a mosquito population is exposed to a particular insecticide, some individual members having resistant genes to this insecticide will survive and reproduce until the resistant allele becomes almost fixed (Corbel and N’Guessan, 2013). Insecticide resistance is mediated by behavioural, metabolic or physiological factors that result from: reduction in insecticide penetration, an increased metabolism of insecticide by metabolic enzymes and/or modification of the insecticide target site (WHO, 1998; IRAC, 2010).

Insecticide resistance is, however, not uniformly distributed among vector species, and can greatly vary from one village, province, country, region and continent to another (IRAC, 2010). Controlling malaria vector mosquitoes through the use of IRS and LLINs is essential to reduce the disease burden. The substantial increase in insecticides application in public health targeting malaria vector mosquitoes, agriculture for crop pests and household level to control domestic pests has resulted in increasing resistance among malaria vectors because of the selection pressure placed on resistant genes (WHO, 2013a).

Following wide use of insecticides in agriculture, public health and at household level, cases of insecticide resistance involving organochlorine, pyrethroid, organophosphate, carbamate insecticides were reported (Brown, 1986; Metcalf, 1999; Munhenga et al., 2008; Masendu et al., 2005; Hunt et al., 2010; Choi et al., 2014; Brooke et al., 2015). The first case of DDT resistance was reported in *An. sacharovi* in Greece in 1953 and was followed by dieldrin resistance in 1954 (Livadas and Georgopoulos, 1953). The onset of insecticide resistance was noticeable by deterioration in malaria control that was characterised by sporadic epidemics of the disease (Brown et al., 1976). In India, house-spraying with DDT and lindane faced serious challenges following resistance of the principal malaria vector *An. culicifacies* to dieldrin in 1958, DDT in 1959, resulting in both lindane and DDT failing to control outbreaks of malaria (Patel et al., 1958; Brown et al., 1976). The experience in Pakistan was similar with DDT resistance appearing in the 1960s and 1970s. The importance of the resistance was not recognised until outbreaks of malaria began in 1969 and neither DDT nor lindane was effective. By 1975, malaria
cases rapidly rose to about 100 million from 9,500 reported in 1961 (Metcalf, 1989). In Sri Lanka, the primary malaria vector *An. culicifacies* was reported to be resistant to DDT, dieldrin, organophosphates, carbamates and pyrethroids, resulting in severe epidemic of malaria in 1968 (Rawlings *et al.*, 1985; Brown, 1986).

The highest levels of insecticide resistance were reported in Africa where malaria burden is still the highest in the world (WHO, 2011). In Africa, after insecticide resistance was initially found in *An. gambiae* in 1967 in Burkina Faso, DDT resistance was reported in neighbouring countries including Cote d’Ivoire, Nigeria and Mali (Hamon *et al.*, 1968), as well as in most of central and eastern African countries (Corbel and N'Guessan, 2013). Strong association was observed between the level of DDT resistance in malaria vectors and the amount of DDT use for cotton protection (Chandre *et al.*, 1999). Meanwhile the first case of dieldrin resistance was reported in 1954 in Nigeria, only a few months after its introduction for use in malaria control, and resistance to dieldrin is still widespread in *Anopheles* populations despite its withdrawal from public health use for many decades (Wondji *et al.*, 2011).

Although organochlorine compounds have been used for a long time in Zimbabwe, there have been few reports of resistance in major malaria vector mosquitoes (Munhenga *et al.*, 2008). Three reports of organochlorine resistance have been observed in Zimbabwe; in Chiredzi involving BHC (Green, 1982), and in Gokwe involving DDT (Masendu *et al.*, 2005; Munhenga *et al.*, 2008).

While the occurrence of insecticide resistance in malaria vectors is not a ‘new’ trait, the speed at which resistance to pyrethroids, the gold standard insecticides for IRS and LLINs has recently developed in the mosquito populations is worrying as it may reverse the gains made in malaria control in recent years in Africa (Corbel and N'Guessan, 2013). Globally, pyrethroid resistance is high in *An. gambiae s.l.* in West Africa including Benin (Corbel *et al.*, 2007), Burkina Faso (Diabate, 2002), Ghana (Yawson *et al.*, 2004), Mali (Fanello *et al.*, 2003), Niger (Czeher *et al.*, 2008), Nigeria (Awolola *et al.*, 2002) and Cote d’Ivoire (Koffi *et al.*, 2012). In West Africa, pyrethroid resistance is predominant in *An. gambiae* than *An. arabiensis* (Corbel and N'Guessan, 2013).
In Central Africa, pyrethroid resistance is wide-spread in *An. gambiae s.l.* in Cameroon (Ndjemai *et al.*, 2009), Chad (Ranson *et al.*, 2009), Gabon (Mourou *et al.*, 2010), Equatorial Guinea (Sharp *et al.*, 2007) and Sudan (Himeidan *et al.*, 2007). Additionally, in Chad, North Cameroon and Sudan, pyrethroid resistance is also present in *An. arabiensis*, which is consistent with the higher prevalence of this mosquito species in more arid localities with higher mean annual temperatures (Costantini *et al.*, 2009). In East, Central and Southern Africa, *An. gambiae* and *An. arabiensis* are mostly susceptible to pyrethroids in Tanzania (Kabula *et al.*, 2012), Mozambique (Coleman *et al.*, 2008) and Madagascar (Ratovonjato *et al.*, 2003), but highly resistant in eastern Uganda (Ramphul *et al.*, 2009), Ethiopia (Abate and Hadis, 2011), Kenya (Ochomo *et al.*, 2012), Zambia (Chanda *et al.*, 2011), South Africa (Mouatcho *et al.*, 2009) and the Gwave area in Gokwe District of Midlands Province, Zimbabwe (Munhenga *et al.*, 2008).

Literature on *An. funestus* has shown that most of the pyrethroid resistance reports have come from South Africa (Mouatcho *et al.*, 2007; Brooke *et al.*, 2013) and Mozambique (Cuamba *et al.*, 2010), most probably because *An. funestus* is the principal malaria vector in these two countries. The data available in several African countries is limited, partly owing to the difficulty of rearing *An. funestus* species in the laboratory (Dia *et al.*, 2013). However, in Mandeya ward in Mutasa District of Manicaland Province, Choi *et al.* (2014) reported pyrethroid and carbamate resistance in *An. funestus* but the same species was susceptible to DDT and organophosphates.

Insecticide resistance in malaria vector mosquitoes is increasing worldwide owing to the increasing selection pressure on mosquito populations mostly caused by the increased use of insecticides in public health, agriculture and at household levels (Verhaeghen *et al.*, 2010). Consistent and longitudinal monitoring surveys are important to identify and address the current changes in resistance and to design appropriate strategies for better insecticide resistance management in malaria vector mosquitoes in Zimbabwe, especially in Mutare and Mutasa Districts.

Various mechanisms enable insects to resist the action of insecticides. The main categories include metabolic resistance, target-site resistance, reduced penetration and behavioural resistance (Fukuto, 1990; IRAC, 2010). These resistance mechanisms are briefly described in the following sections.
2.9.1 Metabolic resistance

Metabolic resistance is the most common resistance mechanism that occurs in insects. This mechanism is centred on the enzyme systems which all insects possess to detoxify (Plate 5) naturally occurring foreign materials which gain entrance into their bodies (Fukuto, 1990; IRAC, 2010). Three groups of enzymes, including esterases, monooxygenases (P450) and glutathione-S-transferases confer resistance to malaria vector mosquitoes. These enzyme systems are usually enhanced in resistant mosquito strains enabling them to metabolise or degrade insecticides before they are able to exert a toxic effect (IRAC, 2010). One of the most common metabolic resistance mechanisms is that of elevated levels or activity of esterase enzymes, which breaks ester bonds of insecticides. The enzyme families contain multiple enzymes with broad covering substrate specificities, and one member of the family might have the capabilities of breaking limited number of insecticides. The degree of resistance conferred may differ from low to high and from one compound to the other (IRAC, 2010; Corbel and N’Guessana, 2013).

Metabolic resistance mechanisms have been reported in mosquito populations for all major classes of insecticides currently applied for vector control, including organochlorines, pyrethroids, organophosphates and carbamates (Brogdon and McAllister, 1998; WHO, 2013). Work by Vaughan et al. (1997) demonstrated that Culex quinquefasciatus was resistant to a broad range of organophosphate compounds. In this species, multiple copies of esterase-gene were found which led to the overproduction of this type of enzyme, resulting in the resistance in this species. On the contrary, a number of Anopheles species including An. culicifacies, An. stephensi and An. arabiensis have shown non-elevated esterases mechanism that confers resistance specifically to malathion through increased rates of metabolism (Brogdon and McAllister, 1998). Malathion resistance in Anopheles species was therefore associated with altered form of esterases that specifically breaks the molecule at a much faster rate than that in susceptible counterparts (Hemingway, 1983).

2.9.2 Target-site resistance

The second most common resistance mechanism observed in vector mosquitoes is target-site resistance. Insecticides generally act on a specific site in the body of the mosquito, within the central nervous system (IRAC, 2010; Corbel and N’Guessana, 2013). The site of action can be
modified (Plate 5) in resistant strains of mosquito species such that the insecticide no longer binds effectively. Reduced sensitivity of the target receptors to insecticide is a genetic mutation, resulting in the mosquito being unaffected or less affected by the insecticide than susceptible species (IRAC, 2010). The target-site for organophosphates and carbamates is an enzyme AChE in the nerve cell synapses. Several mutated forms of AChE (also called MACE [modified acetylcholinesterase]) have been found in mosquitoes (Fournier, 2005) which result in reduced sensitivity to inhibition of the enzyme by these insecticides (Weill et al., 2003).

![Plate 5: Schematic diagram of potential behavioural and physiological changes associated with insecticide resistance in malaria vectors: (a) susceptible insects; (b) resistant insects (Corbel and N'Guessan, 2013)](image)

Similarly, mutations in the amino acid sequence in the voltage-gated sodium channels of nerve cell membranes leads to a reduction in the sensitivity of the channels to the binding of DDT and pyrethroid insecticides (Davies et al., 2007). Alterations in the target-site that cause resistance to
insecticides are referred to as knockdown resistance (kdr) and this refers to the mosquitoes with traits to withstand prolonged exposure to insecticides without being knocked-down (Donnelly et al., 2009). Reduced susceptibility to pyrethroids conferred by kdr mutations has been confirmed in An. gambiae in West, Central and East Africa (IRAC, 2010).

2.9.3 Reduced penetration

Reduced penetration (Plate 5) is a relatively minor resistance mechanism and is associated with modification in the mosquito cuticle or digestive tract linings that prevent or slow the absorption or penetration of insecticides (IRAC, 2010). The resistance mechanism is not specific and can involve a broad range of insecticides, especially DDT and pyrethroids (Corbel and N'Guessan, 2013). However, for malaria vector control, where insecticides are applied on nets and indoor surfaces, the uptake of insecticides which is mostly through the appendages will be reduced following increase in the thickness of the tarsal cuticle (Corbel and N'Guessan, 2013).

2.9.4 Behavioural resistance

Insecticide resistance in mosquito populations is not always based on biochemical mechanisms like metabolic detoxification or target-site mutations, but may also be conferred by behavioural modification (Plate 5) in response to prolonged exposure to an insecticide (IRAC, 2010; Corbel and N'Guessan, 2013). Behavioural resistance describes any modification in insect behaviour that helps to avoid the lethal effects of insecticides. The resistance does not have the same importance as physiological resistance, but might be a contributing factor to the avoidance of lethal doses of an insecticide applied through IRS or LLINs (Roberts et al., 1997).

The first study on the irritant effect of DDT was conducted using An. quadrimaculatus where mosquitoes were observed to be irritated shortly after making contact with the treated surfaces resulting in rapid escape responses from treated structures before taking blood meal (Gahan and Lindquis, 1945). Recent findings in Tanzania showed a shift in An. funestus from indoor to outdoor biting in relation to increasing coverage of pyrethroid-treated nets (Russell et al., 2011). This type of response can be categorised into direct contact excitation (irritancy) and non-contact (repellency) that is used when mosquitoes move away from the insecticide-treated surfaces prior direct contact (Chareonviriyaphap et al., 1997).
2.9.5 Cross resistance

Cross resistance occurs when a resistance mechanism that allows insects to resist one insecticide, also confers resistance to compounds within the same class, and may occur between insecticide classes (IRAC, 2010). The trait of cross-resistance is common in vector populations. Although DDT and pyrethroids are chemically unrelated, both insecticides act on the same target-site, the voltage-gated sodium channel and resistance to DDT has resulted in resistance to pyrethroids in several mosquito species due to the kdr mutation at the target site (IRAC, 2010). Pyrethroid resistance with cross-resistance to DDT was first reported in An. gambiae in Cote d’Ivoire (Elissa et al., 1993) and is now widespread in West Africa (IRAC, 2010). Pyrethroid and DDT cross resistance present a major challenge for malaria vector control in Africa because pyrethroid represent the only class of insecticides approved for treating nets while DDT is commonly used in IRS in several parts of Africa (WHO, 2006b). In contrast, there is lack of cross-resistance between pyrethroids and DDT involving An. funestus species in most countries in Southern Africa, primarily because pyrethroid resistance in this species is mostly conferred fully or partially by monooxygenases (P450) in this region (Coetzee and Koekemoer, 2013). Further, it appears there is no knockdown resistance (kdr) gene in Southern African An. funestus to date (Coetzee and Koekemoer, 2013). Meanwhile, cross-resistance can also occur between organophosphate and carbamate insecticides, resulting from altered AChE.

Some chemical subgroups may have the same mode of action but are chemically different and are less likely to lead to cross resistance. For example, OPs and carbamates subgroups have the same mode of action, DDT and pyrethroids subgroups also have the same mode of action, but there is not always cross resistance between the two groups, especially when metabolic resistance is involved (Corbel and N'Guessan, 2013).

2.9.6 Multiple resistance

Multiple resistance occurs when several different resistance mechanisms are present simultaneously in resistant mosquito populations (IRAC, 2010). The different resistance mechanisms may combine to provide resistance to multiple classes of insecticides. Work by Dabire et al. (2008) has demonstrated multiple resistance in An. gambiae collected from moist savannahs of western Burkina Faso.
In general, the immense challenge in Africa is not to manage and control kdr-resistant mosquitoes only but to deal with the multiple resistant mosquito populations that are not susceptible to different classes of insecticide used in public health. In addition, the occurrence and development of carbamate resistance in some countries especially Benin (Djogbenou et al., 2011), Nigeria (Oduola et al., 2012), and more recently Zimbabwe (Choi et al., 2014) is worrying for insecticide resistance management and alternative insecticides. There is urgent need for innovative strategies to better reduce the vectorial capacity of mosquitoes and hence effectively reduce the burden of malaria in the region, especially in Zimbabwe’s Mutare and Mutasa Districts, through development and use of insecticide resistance management plan for malaria vector control.

2.10 Resistance management strategies

Insecticide resistance management can be undertaken using insecticide-based approaches in conjunction with other insecticidal control intervention methods. The methods which are commonly employed to delay the development of insecticide resistance include rotation, mosaic, mixtures and insecticide resistance in an integrated vector management.
CHAPTER 3
GENERAL METHODS AND MATERIALS

3.1 Study sites

The present study was conducted from May 2013 to December 2014 in the administrative wards of Burma Valley (19°11' S, 32° 48' E; elevation 679 m) and Zindi (18°22' S, 32° 56' E; elevation 766 m) in Mutare and Mutasa Districts, respectively of Manicaland Province (Figure 1). Burma Valley and Zindi are respectively situated about 80 km south and 150 km north of the city of Mutare, the provincial capital of Manicaland Province. The two sites are separated by a distance of about 200 km and both are located along the Zimbabwe-Mozambique border to the eastern part of the country. Both areas are designated field entomological sentinel sites in Manicaland Province and are located in rural areas with similar ecological backgrounds where natural and man-made mosquito breeding habitats exist throughout the year. Malaria transmission is seasonal in the villages surrounding the two study sites, with more cases during the rainy than dry season. Burma Valley is surrounded by 13 villages, six small-scale commercial farms and 16 large-scale commercial farms, with a population of 4,506 people, whereas, Zindi has 19 villages, 23 small-scale commercial farms and one large-scale commercial farm with a population of 9,374 people.

Domestic animals including cattle, goats, chickens, and dogs are kept by many households, as well as pigs and ducks amongst a small minority. Two types of houses are commonly found in the study sites: traditional houses with pole and mud-plastered superstructures and grass thatched roofs, and western-type houses built using burnt bricks bonded by cement mortar. In the latter type of houses, the majority are roofed using asbestos while corrugated iron sheets are used in few households. The main vegetation type in both sites is largely composed of tropical savanna, grassland and woodland characterized by tall grass, trees and bushes, randomly spaced and typically associated with tropical wet and dry climates. The natural vegetation which is commonly green during the rainy season is frequently removed by violent bush burning activities in the dry season.
Both sites have a tropical climate commonly characterized by hot annual mean temperatures ranging from a minimum of 18°C in winter to a maximum of 30°C in summer, with relative humidity and rainfall ranges of 65-85% and 900-1,200 mm, respectively (Taylor and Mutambu, 1986). In addition, the climate presents three distinct seasons: a cold-dry season from April to August with minimum or no rains in April and extremely cold in July, hot and dry season in August to October, completely without meaningful rains, and hot-wet season in November through to March with possible two rainfall peaks in December and February (Taylor and Mutambu, 1986). The rainfall pattern constitutes one season per annum which commonly commences in November and ends in March (Taylor and Mutambu, 1986). Between the villages of Zindi area, several perennial streams flowing from the Nyangani escarpment to Pungwe River create numerous breeding habitats for mosquitoes as the water flows across the villages to Mozambique. Similarly, Burma Valley’s perennial rivers also run through most villages of this ward to Mozambique. In both sites, streams and rivers which are shaded in some areas form wide
stagnant water bodies and marshes which are natural potential breeding sites of malaria vector mosquitoes, especially members of the *An. funestus* group.

Cultivation on the stream and river banks is a common practice in the villages surrounding the study sites, leading to construction of irrigation channels and wells which are man-made potential mosquito breeding sites. Both small and large-scale farming is common, with the majority of the people growing maize, yams and bananas for commercial purposes, and a few households growing sugar cane for domestic purposes and local sale. Eastern Highlands Estates located in Zindi ward together with other few individuals grow tea. A few small and large-scale commercial farms in Burma Valley derive their economy from growing tobacco. Both small and large-scale farmers commonly use pyrethroids, organophosphates and carbamates to protect their crops against different agricultural pests, most probably increasing selection pressure on these insecticides.

Selection of study sites was based on the ecological conditions which provide potential breeding habitats for both *An. gambiae* and *An. funestus* complexes. Malaria transmission in the two sites is regarded as a major public health problem and occurs seasonally, particularly in November through to May, with cases peaking in March/April, and the lowest cases being recorded in June to July (Lukwa *et al*., 2014. The peak malaria months commonly experience epidemics which in some instances become devastating. To prevent malaria transmission in the villages around the two sites, the Zimbabwe NMCP employs several control interventions including IPTp, early diagnosis and effective treatment, as well as IRS, LLINs, selective larval source management and SBCC. Of these interventions, malaria case management, IRS and LLINs have been scaled-up in recent years.

### 3.2 Mosquito collections

Mosquito specimens were collected using larval and adult sampling techniques recommended by WHO (2003). Mosquito larvae were collected using the scooping method, while adult mosquitoes were sampled using techniques that include pyrethrum spray catch (PSC), prokopac aspirator, Centers for Disease Control (CDC) light trap (John W. Hock Ltd, Gainesville FL, USA), exit window trap (EWT), and pit shelter (PS) methods (WHO, 2003; Vazquez-Prokopec...
et al., 2009). All *Anopheles* specimens morphologically identified as either *An. gambiae* complex and/or *An. funestus* group (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987) were transported from the two field entomological sentinel sites to the National Institute of Health Research (NIHR) laboratory in Harare for various assays.

### 3.3 Determination of malaria vector species and their relative abundance

To determine malaria vector species and their relative abundance, mosquito larval sampling technique (WHO, 2003) was the principal method used to collect specimens, complemented by PSC. Weekly surveys were conducted to establish the availability of stagnant water, presence of mosquito larvae and their densities, as well as habitat characteristics from September 2013 to October 2013 in all breeding sites identified. To be considered an aquatic habitat, the water body would have had either anopheline larvae only or anopheline larvae in sympatry with culicine larvae. Types of breeding sites were categorised into human-made and natural in origin and recorded. The man-made habitats were further grouped into yam plantation, shallow well, irrigation channel; whereas the natural breeding sites were classified into rain pool, marsh and river bank (Plate 6).

Larval sampling was conducted using the standard dipping method using a 350 ml mosquito scoop (Bioquip, Gardena, CA, USA) as described by Service (1993). The number of dips taken from each breeding site was dependent on the perimeter of the larval habitat. A maximum of 10 dips were taken from each aquatic habitat. In small habitats where dipping method was not practical, mosquito larvae were collected individually using 7 ml plastic pipettes. Anopheline larval density was calculated by dividing the number of larvae by number of dips. The larvae were then transferred from the dipper by pipetting into a white collecting tray with clear water obtained from the same breeding site, and categorised into different instar stages, followed by counting, morphological identification (Gillies and Coetzee, 1978) and recording. The anopheline larvae were identified morphologically into stage of larval development and recorded as either 1st to 2nd instar (early), 3rd to 4th instar (late) instar or pupae, following taxonomic keys of Gillies and De Meillon (1968) and Gillies and Coetzee (1978).
Plate 6: Types of mosquito aquatic habitats in Burma Valley and Zindi: well (A), yam plantation (B), irrigation channel (C), marsh (D), river bank (E) and temporary rain pool (F)

The larvae were immediately placed in plastic jugs and later taken to entomological field insectaries for rearing to adults according to protocols outlined by WHO (2003). All larvae were kept at room temperature and fed with ground fish food. The adults that emerged (within 1-4 days) were transferred to NIHR laboratory in Harare, where they were killed by anaesthetizing using drops of acetyl acetate placed on a large filter paper that was held above the adults’ container. The specimens were identified into *An. gambiae* complex and *An. funestus* group using morphological characters of Gillies and De Meillon (1968) and Gillies and Coetzee (1987) under x 20 Zeiss light microscope. Afterwards, the identified specimens were collected and stored separately in well-labelled eppendorf tubes containing silica gel prior to identification to sub-species by PCR methods (Scott *et al*., 1993; Koekemoer *et al*., 2002). Other *Anopheles* mosquito species were morphologically identified (Gillies and Coetzee, 1987), recorded and discarded.
3.4 Mosquito resting behaviour studies

Mosquitoes which rest either indoors or outdoors were sampled using PSC, prokopac battery powered aspirator (Vazquez-Prokopec et al., 2009), EWT and PS methods. The sampling was conducted at intervals of two days per month per site from January to December in 2014. Both PSC and prokopac collections were carried out on Monday mornings in Burma Valley and Wednesday mornings in Zindi, whereas EWT and PS sampling was conducted on Tuesday mornings in Burma Valley and Thursday mornings in Zindi. While both PSC and prokopac methods collect indoor resting mosquitoes, the two methods were used concurrently in order to compare the results of the two techniques. Outdoor resting mosquitoes were collected using artificial pit shelters, while exit window traps were set to catch mosquitoes which bite indoors and rest outdoors, as well as gravid mosquitoes leaving structures to find suitable places for oviposition (Pates and Curtis, 2005; Fornadel and Norris, 2008).

Indoor resting mosquito sampling by PSC method was conducted in 10 purposively sampled bedrooms in each site. One of the most important selection criteria was whether one or more people had slept in that room the previous night, and the number of occupants was recorded. The sampled rooms were visited between 06:00 and 10:00 hours for each sampling day. Spray sheet mosquito sampling involves using a pyrethrin space spray to knock down mosquitoes resting inside a house and collecting them on white sheets spread on the floor and other flat surfaces in the structure (WHO, 2003).

A PSC was performed immediately after preparing the house, and the procedure involved removing or completely covering all large pieces of household goods and the floors with white clothing materials. Insecticide was best sprayed from outside of the structure onto the eaves, windows, and door before the mosquito collector quickly entered the house and spraying the interior until the room was filled with fine mist. Once spraying was done, the mosquito collector quickly left the room. All doors and windows remained closed for 10 minutes to allow for mosquito knockdown.

Starting from the doorway, two mosquito collectors picked the white clothing materials one at a time by their corners and collected knocked down mosquitoes outside in daylight using entomological forceps. On windy or rainy days, team members used forceps to collect knocked
down specimens from inside houses with the aid of a hand torch. Collected mosquitoes were placed in a labelled petri dish with a layer of damp cotton wool and filter paper on top of the cotton wool, with a separate petri dish being used for mosquitoes collected from each house. A commercial-grade pyrethroid insecticide, Baygon® (active ingredients d-cyphenothrin, propoxur and improthrin) was used to knock down mosquitoes due to its wide distribution and safety. The number of mosquitoes collected was the total catch for each house.

The proportions of adult mosquitoes collected by PSC method were compared with those from prokopac collections. At each study site, collections of adult mosquitoes using prokopac were conducted between 06:00 and 10:00 hours in 10 purposively selected bedrooms. Collections were made by one worker after grouping the mosquito resting sites inside the rooms into sprayable structures (walls and roof) and unsprayable surfaces (furniture and other household goods) (Plate 7). Further, mosquitoes collected on the walls were categorised according to the wall height above the floor at which they were resting (low: < 1 m, middle: 1-1.5 m, and high: >1.5 m). Aspirations were systematic, starting with sprayable structures and ending with unsprayable surfaces, and specimens were recorded accordingly.
Plate 7: Types of sprayable and unsprayable surfaces respectively in Burma Valley and Zindi: wall (Q), roof (X), furniture (Y) and other household goods (Z)

Adult mosquitoes leaving structures after successful or unsuccessful attempts to feed and gravid mosquitoes that exit houses for oviposition were collected using EWTs (Plate 8) fixed on broken window panes in 10 bedrooms at each site following WHO standard (1975). The houses were purposively selected, especially those huts found situated in environmental conditions that suggests the likelihood of relatively high mosquito density (closeness to breeding sites and relatively high number of people sleeping in the house). One trap was fixed on one house for each selected household.

The traps were installed only on houses without large spaces under the eaves or those dwellings without several missing or broken window panes. In areas where dwellings with these specifications could not be located, the large spaces were reduced by fixing cotton wool or
clothing materials. The EWTs were set between 16:00 and 18:00 hours and all mosquitoes trapped were aspirated between 06:00 and 10:00 hours the following morning.

Outdoor resting mosquitoes were collected using artificial pit shelters (WHO, 1975; Bhatt et al., 1989). A total of 10 pit shelters were dug at each site, conveniently located in most of the villages around the two study areas. In each site, pit shelters were located near houses and cattle shelters, five apiece. Each pit was dug approximately within 50-150 m range between aquatic habitats and each village following techniques described by Thomson (1958) and WHO (1975).

Pits were dug in each study area either under trees or in a shaded area between bushes (Plate 9). The trees were important as they made sure that the opening of the pit was naturally well shaded from above by overhanging branches. Each pit was rectangular in shape, 150-180 cm deep, 120-150 cm long and 90-120 cm wide, with four cavities of 15 cm width and 30 cm depth situated about 45-60 cm from the bottom of the pit. These four little dark cavities or niches form most attractive resting-sites for mosquitoes entering the PS (Thomson, 1958). Each artificial pit shelter

Plate 8: Exit window trap fixed on a window with a broken pane in Burma Valley
was provided with appropriate security facilities to protect against abuse by some people or animals from falling into the pit (WHO, 1975).

Plate 9: Artificial pit shelter in Zindi

3.5 Mosquito biting behaviour studies

Mosquitoes were sampled for two days per month from October 2013 to September 2014 using CDC light traps. Mosquito sampling was divided into two cohorts. One cohort comprised setting up five indoor and five outdoor light traps in the evening and leaving them overnight before collecting trapped mosquitoes the following morning. The other cohort comprised setting up two light traps, one indoor and the other outdoors in the evening and the trapped mosquitoes collected hourly till sunrise of the following morning. In all experiments, light traps were installed by hanging them approximately 1.5 m above the ground close to the feet of a sleeping male local volunteer protected by an insecticide-free mosquito net. The inlet of each trap was set up at the same height as the man’s bed. When the man was sleeping on the floor, the light trap was installed so that the bottom of the collection bag touched the ground (Vazquez-Prokopec et al., 2009). During all indoor collections, no other sleepers were present inside the room. The traps installed outdoors were set approximately 10-20 m away from houses.
The outdoor trap and mosquito net-protected man were under a fruit or wild tree conveniently selected at each household. Sites were chosen from the shades of those big trees at home naturally utilized for resting purposes by local people, especially men during hot weather and/or early night. Two trained teams of two people working in a six-hour rotation collected trapped mosquitoes on hourly basis throughout the night. The teams were responsible for the setting up of the traps at each site (Plate 10), ensuring the traps were correctly installed and switching them on and off at the scheduled time period. Before each day’s field work, each trap had to be thoroughly inspected to ensure that the battery was fully charged and the trap was correctly positioned. At the end of each collection interval, the team ensured that the collection bag was carefully closed and properly stored to secure the catches.

Plate 10: A team of field technician setting up a CDC light trap and an untreated mosquito net for outdoor all night mosquito catches in Burma Valley

In both cohorts, mosquito trapping was conducted from 18:00 to 06:00 hours for two nights per month at randomly-selected houses. Catches were expressed as the mean proportion of mosquitoes collected per trap per night for overnight and mean proportion of mosquitoes per trap per hour for hourly collections. Indoor and outdoor temperature, relative humidity and rainfall readings were recorded at hourly intervals using a digital thermometer, wet and dry bulb thermometer and rain gauge, respectively.
Centers for Disease Control light traps were used as a proxy to measure host searching behaviours of vector mosquitoes since the method is comparable to human landing catches and thus considered an appropriate tool for sampling mosquito vectors that would otherwise bite humans (Lines et al., 1991; Githeko et al., 1994). In addition, it was shown that where use of vector control interventions is high and vector densities are low, CDC light traps can be used to monitor vector human blood rates, assuming that the mosquitoes that entered a trap during any hour were those actively seeking a blood meal, and in most cases would bite human hosts in the same hour and area if the light trap was absent (WHO, 2003; Fornadel et al., 2010).

The gold standard method for determining the human blood rate (HBR) is the human landing catches (HLCs) because mosquitoes are captured by aspiration as they land and attempt to feed on collectors (Beier, 2002). However, in many regions where vector control efforts are underway, use of HLCs may not always be practical due to the increasing ethical and worker safety concerns that this collection method increases the risk of exposure of collectors to infectious mosquitoes (Fornadel et al., 2010). The ethical dilemma is exacerbated in areas of resistance to antimalarials when collectors would otherwise have the opportunity to be protected from infectious bites by sleeping under mosquito nets, and Ethics Review Board in Zimbabwe has deemed this an occupational hazard, leading to rejection to issue ethical clearance for use of HLCs. In addition, the method requires vigilance throughout the night and appears to collect non-standardized data because of variability of the attractiveness and skill of collectors (Lines et al., 1991; Mboera, 2005; Fornadel et al., 2010).

### 3.6 Insecticide susceptibility studies

Indoor resting adult female *An. funestus* group and *An. gambiae* complex for insecticide resistance bioassays were collected between 06:00-10:00 hours in houses in Burma Valley and Zindi areas using prokopac battery powered aspirator (Vazquez-Prokopec et al., 2009) in February 2014. Live female mosquitoes were identified in the field using morphological keys (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). The *An. funestus* populations were divided into two subsamples and were fed with 10% sugar solution. One subsample consisting of only blood-fed samples was immediately assayed in the field insectary using WHO
discriminating dosages of insecticides. The remaining live and all dead specimens were transported to NIHR laboratory in Harare.

Live blood-fed and gravid adult female *An. funestus* were pooled and individually isolated and were allowed to lay eggs on wet filter papers inserted in test tubes. Larvae were reared through to adults in an insectary maintained at 25-27°C and 70-80% relative humidity. F1 adult progeny from each family were used for insecticide and intensity bioassays. Polymerase chain reaction using two legs per mosquito was carried out as described by Koekemoer (2002) to confirm the sibling species of all females that laid eggs. The susceptibility and resistance intensity assays were conducted only on *An. funestus s.s.* F1 progeny females.

Insecticide susceptibility tests were carried out using the standard WHO protocol (WHO, 2013a). Three to five day old non blood-fed adult female *An. funestus* were tested. Batches of 20-25 mosquitoes were exposed to test papers impregnated with DDT (4%), deltamethrin (0.05%), lambda-cyhalothrin 0.05%), etofenprox (0.5%), bendiocarb (0.1%) and pirimiphos methyl (1%). Controls included batches of mosquitoes from each site exposed to untreated papers. The knockdown effect of each insecticide was recorded every 10, 15, 20, 30 and 40 min over the one-hour exposure period. After the one-hour exposure period, mosquitoes were then transferred to a recovery tube and provided with 10% sucrose solution. Final mortality was recorded 24 hours post-exposure.

All batches of insecticide-impregnated papers used were pre-tested on a laboratory strain of *An. gambiae s.s.* maintained at the NIHR insectary, which is known to be susceptible to pyrethroids and DDT. All susceptibility tests were conducted at 26-28°C and 75-80% relative humidity. Dead mosquitoes were counted after a recovery period of 24 h. For each population, there were at least four replicates of each treatment. Results were used only if the mortality in the controls was < 5%, and Abbott’s formula (Abbot, 1925) was used to correct mortalities of more than 5% and less than 20%, and the results were discarded when control mortality was >20% (WHO, 2013a). The WHO’s (2013a) criterion was followed for considering the vector species as being susceptible (mortality 98-100%), potentially resistant (mortality 90-97%) and resistant (mortality < 90%).
In addition to knockdown within one hour, mosquitoes were also exposed for eight hours for a resistance intensity assay. F1 adult progeny female mosquitoes were continuously exposed for eight hours to papers treated with lambda-cyhalothrin (0.05%), deltamethrin (0.05%), etofenprox (0.5%), and bendiocarb (0.1%) and knockdowns were recorded at 5, 10, 15, 20, 30, 40, 50, 60, 80 and 120-min intervals, and hourly thereafter up to eight hours. The 8-hour cut-off was purposely selected as the likely time a mosquito might come into contact with a sprayed wall/surface before or after a taking blood meal (Choi et al., 2014). Knockdown times of KD_{50} (minutes required to knockdown 50% of the mosquitoes) and KD_{95} (minutes required to knockdown 95% of the mosquitoes) were calculated.

### 3.7 Studies to determine blood meal sources and human blood index

All fully blood-fed An. funestus mosquitoes collected were assayed for human, bovine, goat, pig and dog blood antigens simultaneously using cytochrome b-based multiplex PCR for blood meal source identification (Kent and Norris, 2005). Abdomen of each fully-fed mosquito was separated from the head and thorax and ground in 50 μL phosphate-buffered saline (PBS). The final volume was brought to 200 μL with PBS buffer and analysed for blood meal sources using human, bovine, dog, goat, and pig primers. Human blood index (HBI) was calculated by dividing the number of An. funestus mosquitoes with human blood by the total number of An. funestus engorged with blood. Mixed blood meal sources were each treated as two separate blood meal sources in calculation.

### 3.8 Studies to detect sporozoites in infected mosquitoes

Detection of *P. falciparum* sporozoites in female An. funestus was performed using enzyme-linked immunosorbent assay (ELISA) (Wirtz et al., 1987). *Plasmodium falciparum*, the predominant malaria parasite species in Zimbabwe, constitutes 95% of all cases (Lukwa et al., 2014). Once identified, the head and thorax of each mosquito specimen were separated from the body and placed in a 0.5 mL vial. The remainder of each body (wings, legs and abdomens) was stored in eppendorf tubes and kept dry in silica gel for taxonomic analysis. Each mosquito species was tested individually or in pools of up to 10 specimens, provided that they belonged to the same species, came from the same site and collected on the same date (Galardo et al., 2007; Magris et al., 2007). Positives samples in the first ELISA underwent a second test to quantify the
amount of circumsporozoite (CS) protein for each sample. Only the samples that were positive in both tests were eventually considered positive. Sporozoite rate was obtained by dividing the number of *An. funestus* which contained *P. falciparum* sporozoites with the total number of *An. funestus* tested (Beier et al., 1999; WHO, 2003).

### 3.9 Species identification using polymerase chain reaction

Polymerase Chain Reaction (PCR) was carried out using deoxyribonucleic acid (DNA) extracted from legs or wings of each morphologically-identified specimen following the methods of Scott *et al.* (1993) for *An. gambiae* complex and Koekemoer *et al.* (2002) for *An. funestus* group. *Anopheles gambiae* complex specimens were amplified using specific diagnostic primers for *An. gambiae*, *An. arabiensis* and *An. quadriannulatus*, whereas primers for *An. funestus*, *An. leesoni*, *An. vaneedeni*, *An. parensis*, *An. rivulorum*, *An. rivulorum*-like and *An. funestus*-like were used for the *An. funestus* group. The amplified products were visualised on 1% agarose gel stained with ethidium bromide.

### 3.10 Data analysis

Data for the two study sites were tested using two-factor Analysis of Variance (ANOVA) without replication at 5% level of significance. The relative abundance of the species was expressed as the percentage of the total number of *Anopheles* collected. WHO (2013a) guidelines for evaluating susceptibility to insecticides in mosquito populations were followed in which mortality of 98-100% indicated susceptibility, 90-97% suggested potential resistance that needed to be confirmed, and less than 90% indicated resistance.

### 3.11 Ethical considerations

The director for the Zimbabwe National Malaria Control Programme, provincial, district and village authorities as well as household owners and hourly CDC-trapped mosquito collectors were sensitised prior to the study and their verbal consent sought and obtained. All hourly CDC-trapped mosquito collectors were provided with untreated mosquito nets during each study night for the entire sampling period. Three LLINs were donated to each participating mosquito
collector and household following the study. Confidentiality and voluntary participation was assured to the household members who were involved in the study.
CHAPTER 4

MALARIA VECTOR SPECIES COMPOSITION AND RELATIVE ABUNDANCE IN MUTARE AND MUTASA DISTRICTS¹

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4.1 Introduction


Previous studies on An. gambiae complex in Zimbabwe documented five members; An. gambiae, An. coluzzii, An. arabiensis, An. merus and An. quadriannulatus in various combination of sympatry (Masendu et al., 2005; Coetzee et al., 2013). The wide distribution of An. arabiensis, often in association with the non-vector An. quadriannulatus, confirmed its status as the principal human malaria vector in Zimbabwe (Masendu et al., 2005; Munhenga, 2010).

Historically, the An. funestus group consists of at least nine African species: An. funestus sensu stricto, An. rivulorum, An. vaneedeni, An. leesoni, An. confusus, An. fuscivenosus, An. brucei, An. parensis and An. aruni (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Recently, new sibling species including An. rivulorum-like (from West Africa), An. funestus-like (from Malawi) and An. funestus-like-like (from Zambia) (Spillings et al., 2009) have been identified.

In the An. funestus group, An. funestus is the only member that is implicated as an important vector of malaria in sub-Saharan Africa (Coetzee et al., 2000). The sympatric occurrence of An. funestus with minor vectors An. rivulorum and An. leesoni has been found in several countries in Africa (Wilkes et al., 1996; Cohuet et al., 2003) but has not been reported in Zimbabwe. The importance of the above vectors in malaria transmission differs depending on their feeding and resting preference behaviour, seasonal abundance and vectorial capacity (Coluzzi, 1984). These differences therefore, contribute to the varied malaria epidemiological patterns observed in
Africa and, subsequently, different vector behaviour may require different strategies for optimal vector control.

Currently, the two most common vector control strategies are indoor residual spraying (IRS) and long lasting insecticidal nets (LLINs), accounting for almost 60% of global investments in malaria control (WHO, 2015c). Molineaux and Gramiccia (1980) concluded that differences in vector behaviour and insecticide resistance cause the failure of IRS and/or mosquito nets in suppressing malaria transmission in any setting. As such, different vector species may have different insecticide susceptibility status at any given locality and time, thus, insecticides for vector control need to be selected carefully to achieve maximum impact on malaria transmission.

Current vector control studies in Zimbabwe, especially on species composition and relative abundance in relation to malaria control focus mainly on members of the *An. gambiae* complex. This focus on only *An. gambiae* complex and marginalizing the *An. funestus* group may be due to fairly easy larval collection and adaptability of the *An. gambiae* complex to field insectary and laboratory conditions as well as the scarcity of members of the *An. funestus* group. The scarcity of *An. funestus* in Zimbabwe is believed to be associated with consistent implementation of IRS which commenced in the 1940s and expanded in 1980s following national independence in the 1980s and scaled up in the mid-1990s (Munhenga, 2010).

*Anopheles funestus* was last reported about ten years ago by Masendu *et al.* (2005) only at Buffalo Ranch in Chiredzi District. More recently, it was reported in Honde Valley (Choi *et al.*, 2014). Despite continued IRS programmes in place, there is a real possibility of *An. funestus* resurgence as the focus of entomological studies has been almost exclusively on the *An. gambiae* complex. Consequently, the current malaria species composition and densities, especially of *An. funestus* remain unknown in the study areas.

Up-to-date information on species composition and densities of both primary and secondary vectors is crucially needed to properly devise and implement vector control activities to prevent malaria transmission and to assess their effectiveness. Therefore, this study aimed to determine malaria vector species composition and relative abundance in Burma Valley and Zindi in Mutare and Mutasa Districts, respectively.
4.2 Materials and methods

4.2.1 Study areas

The study was undertaken in Burma Valley administrative ward (19°11’S, 32°48’E), Mutare District, and Zindi administrative ward (18°22’S, 32°56’E), Mutasa District (Plate 11). Details of the study sites have been given in section 3.1 in the General Materials and Methods.

Plate 11: Map of Manicaland Province, Zimbabwe, showing the study sites

4.2.2 Mosquito larval sampling and rearing

Weekly physical examinations of natural and human-made mosquito larval habitats were conducted to determine the availability of potential breeding sites for both *An. gambiae sensu lato* (s.l.) and *An. funestus* group for two months for five days per month from September to October 2013. Types of breeding sites were categorised into human-made and natural in origin and recorded. Larval collection was performed once a month from November 2013 to April 2014. Where mosquito larvae were present, 5-10 dips, depending on the size of the habitat, were taken using standard dippers of 350 ml capacity. Samples were classified into 1) group of 1st and 2nd instars, 2) group of 3rd and 4th of instars, and 3) pupae. The instars were identified morphologically using taxonomic keys of Gillies and De Meillon (1968). The number of 1st to 4th
instar larvae of each anopheline species collected per dip represented the larval density in each breeding site.

Immediately after morphological determination of species and larval density assessment, the *Anopheles* larvae were placed in plastic jugs and taken to entomological field laboratories/insectaries for rearing according to WHO (2003) guidelines. All larvae were kept at room temperature and fed with ground fish food. The adults that emerged were transferred to the laboratory at NIHR in Harare. The specimens were killed by anaesthetizing with drops of acetyl acetate placed on a large filter paper that was held above the adults’ container. Emerged adults were identified morphologically into species complexes (*An. gambiae* and *An. funestus*) using taxonomic keys of Gillies and Coetzee (1987). Afterwards, they were individually preserved on silica gel in well-labelled eppendorf tubes prior to polymerase chain reaction (PCR) assays. Other anophelines were morphologically identified (Gillies and Coetzee, 1987), recorded and discarded.

4.2.3 Indoor collections

Indoor resting adult mosquitoes were collected by PSC method (WHO, 2003) in twenty conveniently selected bedrooms in Burma Valley and Zindi, ten bedrooms apiece for one day per month from May 2013 to April 2014. The selected bedrooms were visited between 06:30 and 10:00 hours every study day to collect adult mosquitoes. Anophelines were sorted and identified morphologically into *An. gambiae s.l.* and *An. funestus* group as well as other anophelines (Gillies and Coetzee, 1987). Female specimens were preserved in labelled eppendorf tubes containing silica gel as drying agent waiting further processing at NIHR. Males were recorded and discarded.

4.2.4 Polymerase chain reaction species identification

Polymerase Chain Reaction was carried out using deoxyribonucleic acid (DNA) extracted from two legs of each morphologically identified specimen. *Anopheles gambiae s.l.* specimens were PCR-assayed using a protocol described by Scott *et al.* (1993) and amplified using specific diagnostic primers for *An. gambiae, An. arabiensis* and *An. quadriannulatus*. As for *An. funestus* group, samples were assayed following the methods of Koekemoer *et al.* (2002) with minor
modifications as detailed by Spillings et al. (2009) using primers for *An. funestus*, *An. leesoni*, *An. vaneedeni*, *An. parensis*, *An. rivulorum*, *An. rivulorum*-like and *An. funestus*-like. The results of PCR amplification were visualized on 1% agarose gel by ethidium bromide staining.

4.2.5 Data analysis

Data were analysed using analysis of variance (ANOVA) at 95% confidence limit. The relative abundance of the species was expressed as the percentage of the total number of *Anopheles* collected.

4.3 Results

4.3.1 Larval habitat census

A total of 42 habitats positive for breeding of aquatic stages of mosquitoes were identified in the study areas (Plate 12). Rain pools were temporary, shallow wells, yam plantations and river banks were semi-permanent, while irrigation channels as well as marshes were permanent mosquito habitats. Of these breeding sites, 23 were in Burma Valley and 19 in Zindi. For both study sites, the overwhelming majority of the anopheline-positive habitats were human-made (88.1%, 37/42) with the remainder natural in origin (11.9%, 5/42). Chances of sampling anopheline larvae were higher in irrigation channels in Zindi, but highly heterogeneous in yam plantations in Burma Valley. All larval habitats were located within 2 km of the homesteads of both study sites.
Plate 12: Types of mosquito breeding habitats in Burma Valley and Zindi, Mutare and Mutasa Districts respectively, Zimbabwe: shallow well (A), yam plantation (B), irrigation channel (C), rain pool (D), marsh (E) and river bank (F)

4.3.2 Larval habitat support and relative abundance

Habitat support for larval development differed at the two study sites. In Burma Valley, 34.8% (8/23) of the habitats had only anopheline, while 17.4% (4/23) had only culicine, and were visited 12 times. In Zindi, 15.8% (3/19) of the habitats had anopheline only and 36.8% (7/19) had only culicine, and were visited 10 times. This gave a combined total of 22 longitudinal samples in 12 months for the two study areas. Sympatry in anopheline and culicine larvae was found in 47.8% (11/23) of the habitats in Burma Valley and 47.4% (9/19) in Zindi, suggesting that the mosquito larvae from the subfamilies Anophelinae and Culicinae coexist in almost half of the habitats.

A total of 3,227 immature stages of anopheline larvae (2,438 early instars, 789 late instar) were collected in Burma Valley and 1,621 (872 early instars, 749 late instars) in Zindi. Pupae were observed in 28.6% (12/42) of larvae-positive sites. *Anopheles* larvae were more abundant in...
Burma Valley than in Zindi (ANOVA: df = 6; F = 12.11; P < 0.01). The mean density of *Anopheles* larvae over the entire sampling efforts was 4.2 and 1.8 larvae per dip in Burma Valley and Zindi, respectively. The majority of *An. funestus* larvae were collected in irrigation channels, marshes and shallow wells, whilst *An. gambiae s.l.* larvae were found in rain pools, yam plantations and river banks.

### 4.3.3 Species composition of anopheline larvae

Of the approximately 4,848 *Anopheles* larvae collected, a total of 4,690 adult mosquitoes emerged. From these, two malaria species complexes were morphologically identified, namely, *An. funestus* group and *An. gambiae s.l.* The *An. funestus* group accounted for (1.9%, 87/4,690) of the adults, while *An. gambiae s.l.* constituted (0.2%, 10/4,690), with *An. pretoriensis*, a non-malaria vector species contributing the majority (97.9%, 4,593/4,690) in both study sites. Overall, the densities of adults of the *An. funestus* group and *An. gambiae s.l.* that emerged from the larval collections were low, but the *An. funestus* group was about eight times more abundant than *An. gambiae s.l.*

### 4.3.4 Species composition and abundance of adult *Anopheles* mosquitoes

A total of 5,625 *Anopheles* adults, including males and females were collected from larval sampling and indoors through PSC methods (Table 2). Of the total collections, *An. pretoriensis* was the most abundant while the remainder was shared between the *An. funestus* group and *An. gambiae s.l.* Overall, more anopheline mosquitoes were collected from aquatic stages (i.e. reared from larvae) than indoors (i.e. as adults), but indoor collections by PSC method had more malaria vector mosquitoes than larval collection. Of note, the results showed that the combined female *An. funestus* group and *An. gambiae s.l.* were approximately five times more abundant than males (Table 2).
Table 2: Percentage mosquito species composition sampled in Burma Valley and Zindi as identified by morphological means

<table>
<thead>
<tr>
<th>Sampling method‡</th>
<th>Study area</th>
<th>N§</th>
<th>An. funestus group</th>
<th>An. gambiae s.l.</th>
<th>An. pretoriensis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>PSC</td>
<td>Burma Valley</td>
<td>795</td>
<td>16.0</td>
<td>80.6</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>140</td>
<td>9.3</td>
<td>87.1</td>
<td>0.7</td>
</tr>
<tr>
<td>RFL</td>
<td>Burma Valley</td>
<td>3,141</td>
<td>0.3</td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>1,549</td>
<td>0.1</td>
<td>2.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>5,625</td>
<td>2.6</td>
<td>14.9</td>
<td>0.2</td>
</tr>
</tbody>
</table>

§ Total number of mosquitoes
‡ PSC = Pyrethrum spray catch; RFL = Reared from larvae

4.3.5 Species composition and abundance of female adult malaria vectors

Data on the species composition and relative abundance of female malaria vector mosquitoes are presented in Table 3. Altogether, more than 800 females of the An. funestus group and An. gambiae s.l. mosquitoes were collected. As observed from Table 3, there were no significant differences in species composition in the two sites (ANOVA: d.f = 1; $F = 3.25; P = 0.17$) though the relative abundance of An. funestus group and An. gambiae s.l. was more in Burma Valley than in Zindi (ANOVA: d.f = 9; $F = 3.65; P < 0.01$). All in all, members of the An. funestus group were 27 times more abundant than An. gambiae s.l. in both sites (Table 3).
Table 3: Species composition (%) of *An. funestus* group and *An. gambiae s.l.* adult females by sampling method in Burma Valley and Zindi

<table>
<thead>
<tr>
<th>Sampling method‡</th>
<th>Study area</th>
<th>N§</th>
<th><em>An. funestus</em> group</th>
<th><em>An. gambiae s.l.</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>PSC</td>
<td>Burma Valley</td>
<td>663</td>
<td>96.7</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>126</td>
<td>96.8</td>
<td>3.2</td>
</tr>
<tr>
<td>RFL</td>
<td>Burma Valley</td>
<td>37</td>
<td>91.9</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>45</td>
<td>95.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>871</td>
<td>96.4</td>
<td>3.6</td>
</tr>
</tbody>
</table>

§ Total number of mosquitoes
‡PSC = Pyrethrum spray catch; RFL = Reared from larvae

4.3.6 PCR analysis of *An. funestus* group sibling species

From a total of 840 females that had been morphologically identified as members of the *An. funestus* group, 294 specimens were PCR assayed. Two sibling species were identified: *An. funestus* (90.8%, 267/294) at a position of 505 base pairs (bp) and *An. leesoni* (5.1%, 15/294) at 146 bp (Plate 13). Twelve specimens (4.1%, 12/294) failed to amplify after two replicates, though the positive control amplified successfully.

4.3.7 Polymerase chain reaction based assays to identify sibling species of *An. gambiae s.l.*

Out of a total of 31 females positively identified to be *An. gambiae s.l.*, 48.4% (15/31) were identified as *An. quadriannulatus* while 41.9% (13/31) were *An. arabiensis* (Plate 14). About 9.7% (3/31) of the *An. gambiae s.l.* tested could not be identified to specific species by PCR using the then available primers specific for *An. gambiae*, *An. arabiensis* and *An. quadriannulatus* sibling species.
Plate 13: Identification of members of the *An. funestus* group from Burma Valley and Zindi: negative control (lane A), positive control (lane B), 100 base pair molecular ladder (lane I), *An. funestus* (505 base pair) (lanes C, D, F and R), *An. leesoni* (146 base pair) (lane G, H, J, M, N, and O), *An. funestus* and *An. leesoni* mixed DNA (lane K), no amplification (lanes E, L, P, Q, S, and T)

Plate 14: Species identification of members of *An. gambiae s.l.* from Burma Valley and Zindi: *An. quadriannulatus* (150 base pair) (lanes 1, 3-8, 10, 12-14 and 16), *An. arabiensis* (315 base pair) (lanes 2, 11 and 15), 100 base pair molecular marker (lane 9)
4.3.8 Percentage species composition and abundance of *An. funestus* and *An. arabiensis* in Burma Valley and Zindi

*An. funestus* and *An. arabiensis* sibling species were present in both study sites, though both species were less abundant in Zindi than in Burma Valley (Table 4). The relative abundance of *An. arabiensis* was generally low in both sites. Overall, *An. funestus* was more abundant than *An. arabiensis* in both sites (ANOVA: df = 2; F = 14.20; P = 0.02).

Table 4: Species composition of *An. funestus* and *An. arabiensis* in Burma Valley and Zindi

<table>
<thead>
<tr>
<th>Study area</th>
<th>N§</th>
<th>An. funestus</th>
<th>An. arabiensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burma Valley</td>
<td>169</td>
<td>95.3 (161)</td>
<td>4.7 (8)</td>
</tr>
<tr>
<td>Zindi</td>
<td>111</td>
<td>95.5 (106)</td>
<td>4.5 (5)</td>
</tr>
<tr>
<td>Total</td>
<td>280</td>
<td>95.4 (267)</td>
<td>4.6 (13)</td>
</tr>
</tbody>
</table>

4.4 Discussion

The study showed the presence of two *Anopheles* complexes: *An. gambiae* and *An. funestus* in Burma Valley and Zindi. Both complexes contain members of important malaria vectors in sub-Saharan Africa (Coetzee *et al.*, 2000). Morphologically identified adults reared from larval collections revealed the presence of predominantly *An. pretoriensis* (a non-malaria vector) and relatively low numbers of adults of the *An. funestus* group and *An. gambiae s.l.* The members of the *An. funestus* group were, however, relatively more abundant than members of *An. gambiae s.l.* These findings are consistent with previous studies in Zimbabwe that demonstrated dominance of *An. pretoriensis* reared from larvae over other anopheline species (Masendu *et al.*, 2005).

This study clearly showed that malaria vectors belonging to the *An. funestus* group and *An. gambiae s.l.* breed in sympatry in the study sites. Most of the mosquito larval habitats in the study areas are human-made; suggesting that malaria transmission in the study areas is derived mainly from human modification and manipulation of the ecosystem. While the irrigation facilities are important to sustain food security, mitigating measures need to be put in place to minimize breeding of vector mosquitoes.
In the present study, members of the *An. funestus* group were more abundant than members of *An. gambiae s.l.* from both adult and larval collections. Previous work by Mpofu (1985), Taylor and Mutambu (1986), and Masendu *et al.* (2005) had shown members of *An. gambiae s.l.* to be the more abundant malaria vector in Zimbabwe.

Using PCR, the present study identified two species: *An. funestus* and *An. arabiensis* that transmit malaria in the study sites. PCR identification of sibling species of the *An. funestus* group and *An. gambiae s.l.* is of great importance to the malaria control programme in Zimbabwe. Molecular analysis of the *An. funestus* group established the presence of *An. funestus* and *An. leesoni*. This is in agreement with studies by Oyewole *et al.* (2005) but in contrast with other previous studies in Africa which recorded *An. funestus* in sympathy with minor vectors *An. rivulorum* and *An. leesoni* (Wilkes *et al.*, 1996). The failure to amplify 5.1% of the *An. funestus* group could be due to morphological misidentification of some specimens and/or emergence of other sibling species. Meanwhile, PCR assay of *An. gambiae s.l.* also revealed two common members: *An. quadriannulatus* and *An. arabiensis*, but could not exclude the possibility that other members of the complex might be amongst the specimens (9.7%) that failed to amplify. It is likely that the unidentified *An. gambiae s.l.* could be *An. merus* as the protocol by Scott *et al.* (1993) used in this study did not include primers to identify *An. merus* which Masendu *et al.* (2005) reported as being common in some parts of Zimbabwe.

Polymerase chain reaction assays revealed *An. funestus* to be more abundant than *An. arabiensis*, confirming the status of the former as a major malaria vector mosquito in both sites. This is in sharp contrast with results from work by Mpofu (1985), Taylor and Mutambu (1986) and Masendu *et al.* (2005) which had shown *An. arabiensis* to be a primary vector while *An. gambiae* and *An. funestus* were secondary vectors in Zimbabwe. The sharp increase in *An. funestus* population in the presence of IRS implementation in this study sites cannot be explained in this study. It is more likely that pyrethroid-resistant *An. funestus* survived undetected while *An. arabiensis* and to a larger extent, *An. gambiae*, remained susceptible to pyrethroids which have been used for IRS and treatment of LLINs over the years in the study areas.

In general, species replacement for various reasons, especially as a result of IRS and LLINs is not a new phenomenon (Kitau *et al.*, 2012). Recent data from East Africa showed changes in
sibling species following the scaling-up of ITNs/LLINs, with *An. arabiensis* becoming the dominant species in habitats that previously supported sympatric *An. gambiae* and *An. arabiensis* populations (Kitau et al., 2012). A similar change in species composition and abundance was reported during the implementation of IRS in Zimbabwe; example being the observation of *An. funestus* in most parts of Zimbabwe by Mpofu (1985), which was isolated in later studies by Masendu et al. (2005) only at Buffalo Ranch in Chiredzi District.

As revealed by the findings of this study, heterogeneity of vector species composition and dominance of *An. funestus* is of major significance in malaria control. The results have important implications for malaria epidemiology and control given that *An. funestus* is a more efficient vector than *An. arabiensis*. Low densities of *An. funestus* have the potential to sharply increase levels of malaria transmission (Morgan et al., 2010).

This study also revealed that there is information gap on the resting, biting and insecticide susceptibility status of malaria vectors in the study sites. The information is crucial for planning, implementation and evaluating malaria vector control strategies. Additionally, the emergence/upsurge of *An. funestus* in areas under IRS and LLINs use requires urgent attention to prevent possible malaria outbreaks. Mitigation measures for irrigation projects or farming should be put in place to minimize malaria vector breeding.
CHAPTER 5

INSIGHTS INTO RESTING BEHAVIOUR OF MALARIA VECTOR MOSQUITOES IN MUTARE AND MUTASA DISTRICTS

This chapter has been published as: Sande, S., Zimba, M., Chinwada, P., Masendu, H.T. and Makuwaza, A. 2016. Insights into resting behavior of malaria vector mosquitoes in Mutare and Mutasa districts of Manicaland province, Zimbabwe. doi: 10.1093/jme/tjw044.
5.1 Introduction

*Anopheles funestus* Giles is one of the most known malaria vector mosquitoes in sub-Saharan Africa (Gillies and De Meillon, 1968). In addition, even in the presence of other efficient vector species like *An. gambiae* s.s. Giles and *An. arabiensis* Patton; *An. funestus* s.s. contributes a large proportion of infectious bites that maintain malaria transmission among different communities.

Malaria remains the most important vector-borne public health problem globally, with the major burden occurring in the African Region (WHO, 2014a; WHO, 2015a; WHO and UNICEF, 2015). In 2010, the World Health Organization estimated that approximately 99 countries still had ongoing high burden of malaria annually, 219 million cases (range 154-289 million), with the disease killing an estimated 660,000 people. Approximately, 81% of cases and 91% of the deaths occurred in the WHO African Region, 86% of the victims were children under five years of age (WHO, 2015b). In Zimbabwe, malaria continues to be the major parasitic disease of public health importance causing morbidity and mortality despite intensive prevention and control efforts.

Vector control is currently the major intervention for global malaria prevention, control and elimination (WHO, 2015b). It remains critical for the reduction in malaria incidence and deaths. Presently, indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) are the two major vector control strategies that contribute to the prevention and control of malaria transmission (WHO, 2010b). Indoor spraying of houses with residual insecticides reduces the longevity of indoor resting anopheline mosquitoes, greatly limiting the probability of malaria transmission. The effectiveness of IRS in combating malaria transmission and disease burden was first shown in the 1930s in South Africa (De Meillon, 1936; Park 1936) and India (Covell *et al*., 1938), and late 1940s in Zimbabwe (Munhenga *et al*., 2008; Lukwa *et al*., 2014). During malaria eradication campaign, IRS using dichloro-diphenyl-trichloro-ethane (DDT) was one of the key methods by which malaria was eradicated in the temperate zones and in reducing malaria incidence in India from 75 million cases per annum in the 1930s to 110,000 per year in the 1960s (WHO, 2010b).
The efficacy of IRS as a vector control tool depends strongly on the behaviour of the vector mosquito, and is more effective against vectors which rest indoors (endophily). This characteristic is typically observed for the major malaria vectors in sub-Saharan Africa and has contributed greatly to the success of IRS as one of the leading malaria control strategies in this region (Pates and Curtis, 2005; O’Meara et al., 2010). However, most of the mosquito species which are in close contact with people and domestic animals are usually found resting inside houses or in animal shelters, therefore giving the impression that these are their only resting places (Bhatt et al., 1989). The resting behaviour of vectors could be much more variable, with some species expressing tendency to rest outdoors (exophily), indoors (endophily), either on sprayable locations (walls and roofs) or unsprayable surfaces (household objects). The tendencies by vector mosquitoes to rest outdoors and on unsprayable locations inside houses limit the effectiveness of IRS as a strategy for malaria prevention (Durnez and Coosemans, 2013).

For the past five years, indoor spraying of roofs higher than 3.5 m from the floor level was rarely accomplished in Burma Valley and Zindi villages in Mutare and Mutasa Districts respectively, following non provision of spray extension lances by the National Malaria Control Programme (NMCP). Spray extension lances enable spraying of surfaces not usually accessible with standard lances provided as components of the sprayers. The unsprayed surfaces provide alternative resting places for vectors in these sprayed structures, thus undermining the effectiveness of IRS in fighting against malaria transmission.

The resting behaviour of mosquitoes may vary considerably between different species, and even in the same species in different areas and seasons (Bhatt et al., 1989). However, behavioural change by malaria vector mosquitoes, so that a larger proportion of the species rests outdoors or indoors on unsprayed surfaces in sprayed structures, threatens to reverse the gains made by IRS in malaria control (Durnez and Coosemans, 2013).

Although An. funestus.s.s. and An. gambiae s.s. are naturally endophilic species in Africa, behavioural resistance in vectors in some countries has arisen in response to prolonged spraying programmes, especially using DDT and/or pyrethroids (Pates and Curtis, 2005). This may result from an immediate response to the irritant and/or repellent insecticides, particularly DDT or
pyrethroids, or it may be a genetic trait evolved selection pressure from the presence of insecticides in the houses (Pates and Curtis, 2005). The irritant effect of insecticide was demonstrated in Burkina Faso where a 94% exit rate of *An. funestus* and *An. gambiae* from pyrethroid-treated huts was observed (Darriet, 1991).

Not much is known about the response of the vector mosquitoes to prolonged IRS programme in Burma Valley and Zindi areas in Mutare and Mutasa Districts, respectively. Despite the number of studies on resting behaviour of malaria vector mosquitoes in Zimbabwe (Masendu, 1996; Dandalo, 2007), those that looked at their resting behaviour in Mutare and Mutasa Districts are scarce. Most of the researchers on malaria and vectors have concentrated in Gokwe District in the Midlands Province. Information on the resting behaviour of anopheline mosquitoes and its relationship to major vector control tools is of great importance in malaria prevention and control. The present work aimed at providing detailed information on the resting behaviour of the adult female *Anopheles* mosquito, following decades of IRS implementation in Mutare and Mutasa Districts. This will help to improve on future application of appropriate modes of malaria control strategies and maximize the use of ever dwindling resources.

### 5.2 Materials and Methods

#### 5.2.1 Study sites

The study was conducted in the villages of Burma Valley (19°11’ S, 32°48’ E), Mutare District, and Zindi (18°22’ S, 32°56’ E), Mutasa District. Study sites details have been given in section 3.1 in the General Materials and Methods.

#### 5.2.2 Mosquito collections

Indoor and outdoor resting mosquitoes were collected at intervals of two days per month per site for one year between January and December 2014 using prokopac battery powered aspirator (Vazquez-Prokopec et al., 2009), pyrethrum spray catch (PSC), exit window trap (EWT), and artificial pit shelter (PS) methods (WHO, 2003). During each collection day, PSC and prokopac collections were performed on Monday mornings in Burma Valley and Wednesday mornings in Zindi, while EWT and PS catches were on Tuesday mornings in Burma Valley and Thursday
mornings in Zindi. Pyrethrum spray catch and prokopac methods collected indoor resting mosquitoes, while outdoor resting specimens were sampled by artificial pit shelters. Exit traps were used to catch mosquito species which enter houses at night, bite and leave soon after feeding without resting indoors as well as gravid mosquitoes leaving dwellings for oviposition (Pates and Curtis, 2005; Fornadel and Norris, 2008).

Indoor resting mosquito collection by PSC method was performed in 10 purposively sampled bedrooms in each site. The sampled rooms were neither sprayed nor issued with LLINs, and were visited between 06:00 and 10:00 hours for each sampling day. The PSC involved removing large furniture items, completely covering the floor and other small household goods with white clothing materials. Insecticide spraying commenced from the outside onto the eaves and doors to drive mosquitoes inside, completing the activity by spraying the entire inside of the room. All doors and windows remained closed for 10 minutes before collection of knocked down mosquitoes. A pyrethroid-insecticide aerosol (commercially marketed as Baygon®) which has the synergist piperonyl butoxide was used. Piperonyl butoxide inhibits oxidase activity. A synergist is a product which does not itself have insecticidal properties, but which, when mixed or applied with insecticides of a particular class, considerably enhance their potency by inhibiting an enzyme that normally acts to detoxify the insecticide (WHO, 2013a).

The percentages of adult mosquitoes collected by PSC were compared with those collected by prokopac method. Mosquito sampling by prokopac also targeted 10 bedrooms in each site, and the selected structures were unsprayed and had not been issued with LLINs. The houses were purposively selected to maximise production, and collection was conducted between 06:00 and 10:00 hours. Mosquito sampling using prokopac was carried out systematically following resting locations inside houses. The resting surfaces were categorized into sprayable locations (walls, roofs) and unsprayable locations (household furniture and other objects). Samples collected were classified into four groups: wall, roof, furniture and other household goods capture stations. Mosquito samples collected on the walls were further classified into specific heights above the floor: low (<1 m), middle (1-1.5 m), and high (>1.5 m), with specimens recorded accordingly.

Adult mosquitoes leaving the houses after successful or unsuccessful attempts to feed, and those which exited dwellings to lay eggs were collected by EWTs fixed on broken window panes in
five sprayed and five unsprayed bedrooms (10 bedrooms) without LLINs at each site following WHO standards (1975). The traps were installed only to houses without large spaces under the eaves, and in areas where dwellings with such specifications were not available; the large spaces were reduced by fixing cotton wool. The EWTs were set between 16:00 and 18:00 hours, with mosquitoes caught aspirated between 06:00 and 10:00 hours the following morning.

Outdoor resting mosquito species were collected from 10 artificial pit shelters (WHO, 1975), at each site, conveniently located in the villages of the two study areas. In each site, five pit shelters were located near human dwellings, while the remaining five were sited close to cattle shelters. Artificial pit shelters were dug in shaded places under trees or thick bushes. Each was rectangular in shape, 1.5-1.8 m deep, 1.2-1.5 m long and 0.9-1.2 m wide. In each of the four vertical sides, about 45-60 cm from the bottom of the pit, a small cavity of 15 cm width and 30 cm depth was dug at right angles to the vertical wall. These cavities have been reported to be the most attractive resting sites for mosquitoes entering the PS (WHO, 1975; Bhatt et al., 1989).

Mosquitoes collected by each method were identified using morphological keys (Gillies and De Meillon, 1968), sorted, counted and recorded. All blood-fed and gravid mosquitoes collected by EWTs were examined under x 20 Zeiss light microscope to confirm their blood digestion stages. The An. funestus group and An. gambiae complex were placed individually into the eppendorf tubes with silica gel to keep them dry, and transported to the National Institute of Health Research (NIHR) laboratory in Harare for sibling species identification using polymerase chain reaction (PCR).

5.2.3 Species identification

Polymerase chain reaction species identification was performed using DNA extracted from legs or wings of a few specimens selected randomly from the lot previously identified using morphological characters following the methods of Koekemoer et al. (2002) in the An. funestus group and Scott et al. (1993) in An. gambiae s.l. mosquitoes.

5.2.4 Data analysis

In this mostly descriptive study, data on mosquito species resting densities were presented in tables and charts made of proportions and percentages of the total number of Anopheles collected
at 95% confidence intervals. Differences in mosquito resting locations and wall heights as well as sprayed and unsprayed structures between the two sites were subjected to statistical analysis using analysis of variance (ANOVA) at the 0.05 level of significance.

5.3 Results

5.3.1 Indoor and outdoor resting behaviour of Anopheles mosquitoes

The relative monthly proportions of anopheline mosquitoes resting indoors and outdoors are shown in Table 5. A total of 592 indoor and outdoor Anopheles mosquitoes were sampled from Burma Valley and Zindi. Anopheles funestus group was the predominant species (96.8%, n = 573), whereas An. gambiae complex mosquitoes were few (3.2%, n=18) of the total Anopheles mosquitoes collected. The results showed distinct monthly variation in indoor and outdoor resting behaviour of An. funestus populations and An. gambiae s.l. in the two areas. The An. funestus group and An. gambiae s.l. had greater endophilic than exophilic tendencies. Of note, the results revealed that combined An. funestus populations and An. gambiae s.l. collected by PSC were approximately five times greater than those caught by artificial PS. However, there were statistical differences between indoor and outdoor resting tendencies in An. funestus populations from January to April, but no differences in the two behaviours from September through to December (Table 5). There were neither indoor nor outdoor collections made during mid-winter (June and July).
Table 5: Comparative monthly percentages of *Anopheles* mosquitoes caught resting both indoors and outdoors in Burma Valley and Zindi using two collection methods during the months January-December 2014

| Month | An. *funestus* group | | | | | An. *gambiae s.l.* | | | | |
|-------|----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|       | N      | PSC   | PS    | *P*-value | N      | PSC   | PS    | *P*-value | N      | PSC   | PS    | *P*-value |
| Jan   | 75     | 86.7  | 13.3  | *0.0017  | 6      | 87.5  | 12.5  | 0.1244  |       |       |       |          |
| Feb   | 90     | 82.2  | 17.8  | *0.0004  | 4      | 100   | 0     | 0.1333  |       |       |       |          |
| Mar   | 154    | 77.9  | 22.1  | *0.0008  | 6      | 90    | 10    | 0.1328  |       |       |       |          |
| Apr   | 136    | 83.8  | 16.2  | *0.0001  | 3      | 100   | 0     | 0.1211  |       |       |       |          |
| May   | 33     | 97    | 3     | 0.1868  | 0      | 0     | 0     | -       |       |       |       |          |
| Jun   | 0      | 0     |       |         | 0      | 0     | 0     | -       |       |       |       |          |
| Jul   | 0      | 0     |       |         | 0      | 0     | 0     | -       |       |       |       |          |
| Aug   | 0      | 0     |       |         | 0      | 0     | 0     | -       |       |       |       |          |
| Sept  | 22     | 100   | 0     | 0.4594  | 0      | 0     | 0     | -       |       |       |       |          |
| Oct   | 27     | 88.9  | 11.1  | 0.2985  | 0      | 0     | 0     | -       |       |       |       |          |
| Nov   | 26     | 84.6  | 15.4  | 0.3177  | 0      | 0     | 0     | -       |       |       |       |          |
| Dec   | 10     | 90    | 10    | 0.2029  | 0      | 0     | 0     | -       |       |       |       |          |
| Total | 573    | 84.1  | 15.9  | -       | 19     | 89.5  | 10.5  | 0.0676  |       |       |       |          |

PSC: pyrethrum spray catches, PS: pit shelter, * significant at *P* < 0.05

5.3.2 Prokopac versus PSC mosquito collection method

The proportions of mosquitoes caught in wet and dry seasons by each collection technique are shown in Table 6. Of the 1,179 mosquitoes captured in both study areas, prokopac aspirator collection method had the most catches (60%, 709/1179) compared with PSC (40%, 470/1179). Overall, the prokopac aspirator collected two times more *An. funestus* mosquitoes per room than the PSC method. There were significant differences in the numbers of *An. funestus* adults collected by prokopac aspirator and PSC methods in wet (*P* = 0.02) and dry (*P* = 0.02) seasons. However, there were no significant differences in the numbers of *An. gambiae* complex, other *Anopheles* mosquitoes and Culicines collected by the two methods in both study sites. All the collections by prokopac aspirator were live, while PSC yielded knocked down mosquitoes. The number of *An. gambiae s.l.* and other *Anopheles* species collected was too small (10 and 9, respectively) to make a meaningful analysis.
Table 6: Percentage of indoor resting mosquitoes collected by Prokopac and PSC techniques in Burma Valley and Zindi during the wet and dry seasons

<table>
<thead>
<tr>
<th>Species</th>
<th>Period</th>
<th>Collection method</th>
<th>Prokopac N</th>
<th>Prokopac %</th>
<th>Density</th>
<th>PSC N</th>
<th>PSC %</th>
<th>Density</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. funestus group</td>
<td>Wet</td>
<td>Prokopac</td>
<td>463</td>
<td>63.8</td>
<td>2.31</td>
<td>262</td>
<td>36.2</td>
<td>1.31</td>
<td>*0.016</td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>Prokopac</td>
<td>310</td>
<td>68.3</td>
<td>1.06</td>
<td>98</td>
<td>31.7</td>
<td>0.49</td>
<td>*0.023</td>
</tr>
<tr>
<td>An. gambiae s.l.</td>
<td>Wet</td>
<td>Prokopac</td>
<td>7</td>
<td>59.6</td>
<td>0.04</td>
<td>4</td>
<td>40.4</td>
<td>0.02</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>Prokopac</td>
<td>3</td>
<td>61.2</td>
<td>0.02</td>
<td>2</td>
<td>38.8</td>
<td>0.01</td>
<td>0.116</td>
</tr>
<tr>
<td>Other Anopheles</td>
<td>Wet</td>
<td>Prokopac</td>
<td>9</td>
<td>70.7</td>
<td>0.05</td>
<td>4</td>
<td>29.3</td>
<td>0.02</td>
<td>0.171</td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>Prokopac</td>
<td>2</td>
<td>66.4</td>
<td>0.01</td>
<td>1</td>
<td>33.6</td>
<td>0.01</td>
<td>0.117</td>
</tr>
<tr>
<td>Culicines</td>
<td>Wet</td>
<td>Prokopac</td>
<td>47</td>
<td>60.5</td>
<td>0.24</td>
<td>31</td>
<td>39.5</td>
<td>0.16</td>
<td>0.381</td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>Prokopac</td>
<td>20</td>
<td>58.1</td>
<td>0.1</td>
<td>14</td>
<td>41.9</td>
<td>0.07</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Wet: wet season Nov-Mar, Dry: dry season Apr-Oct, PSC: pyrethrum spray catches; * significant at P < 0.05

5.3.3 Indoor resting habitats of mosquito species

Table 7 shows variations in the resting behaviour of anopheline mosquitoes on different surfaces inside dwellings. Out of the total number of An. funestus mosquitoes which entered the structures to rest, nearly 90% (n = 546) were collected on sprayable surfaces (walls and roofs), with about 10% (n = 61) found on unsprayable surfaces (furniture and other household goods). Of the sprayable surfaces, nearly 56% (n = 306) of An. funestus mosquitoes selected roofs as resting sites, with wall surfaces constituting about 44% (n = 244) in both study sites. Anopheles funestus collections were 1.25 times more on the roofs than on the walls inside dwellings. A significant difference was observed in the resting habits of An. funestus mosquitoes on the walls and roofs inside houses (P = 0.004). Among the unsprayable surfaces, more An. funestus and An. gambiae s.l. mosquitoes were collected resting on furniture objects than other household goods.

Table 7: Percentages of mosquito species collected indoors according to resting surfaces

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>N</th>
<th>Wall (%)</th>
<th>Roof (%)</th>
<th>Furniture (%)</th>
<th>Other (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. funestus group</td>
<td>607</td>
<td>40.2</td>
<td>50.4</td>
<td>7.2</td>
<td>2.2</td>
</tr>
<tr>
<td>An. gambiae complex</td>
<td>31</td>
<td>35.5</td>
<td>41.9</td>
<td>16.2</td>
<td>6.5</td>
</tr>
<tr>
<td>Other Anopheles species</td>
<td>13</td>
<td>30.8</td>
<td>53.8</td>
<td>7.7</td>
<td>7.7</td>
</tr>
<tr>
<td>Culex species</td>
<td>509</td>
<td>38.3</td>
<td>47.2</td>
<td>9.4</td>
<td>5.1</td>
</tr>
</tbody>
</table>
5.3.4 Wall height indoor resting preferences of mosquito species

There was significant variability in mosquito resting densities on different wall heights from the floors (Table 8). Of the *An. funestus* populations sampled, the majority (about 44%, 108/245) had a greater tendency to rest on wall heights of less than 1 m from the ground, with middle heights being least preferred resting sites (just above 20%, 54/245). Different height of a wall above the floor was found to be a significant factor in the densities of *An. funestus* mosquitoes resting indoors (ANOVA: $df = 2; F = 14.22; P = 0.002$). However, data collected on *An. gambiae s.l.* mosquitoes (2.4%, 11/455) and other *Anopheles* species (0.9%, 4/455) over 12 months of study in both sites could not be meaningfully interpreted.

Table 8: Percentage of indoor resting mosquito according to species and collection height above ground in Burma Valley and Zindi

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>N</th>
<th>Low (&lt; 1)</th>
<th>Middle (1-1.5)</th>
<th>High (&gt; 1.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. funestus</em> group</td>
<td>245</td>
<td>44.1</td>
<td>22.0</td>
<td>33.9</td>
</tr>
<tr>
<td><em>An. gambiae s.l.</em></td>
<td>11</td>
<td>63.6</td>
<td>9.1</td>
<td>27.3</td>
</tr>
<tr>
<td>Other <em>Anopheles</em> species</td>
<td>4</td>
<td>75.0</td>
<td>25.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Culex</em> species</td>
<td>195</td>
<td>39.4</td>
<td>30.3</td>
<td>30.3</td>
</tr>
</tbody>
</table>

5.3.5 Survival rate of exit window trap collections after 24-hour holding period

The results of *Anopheles* mosquitoes collected by exit window traps from sprayed and unsprayed structures in Burma Valley and Zindi sites showed variations in densities, percentage of abdominal appearance, as well as survival rate after 24-hour holding period (Table 9). The percentage of *Anopheles* mosquitoes found dead in the exit traps were lower in unsprayed than sprayed structures. The highest percentage of *An. funestus* mosquitoes found exiting both sprayed and unsprayed dwellings were gravid. Of the gravid *An. funestus* mosquitoes caught, the majority, about 65% (204/312), were collected exiting sprayed structures in both sites. A similar trend was observed in *An. gambiae s.l.*, of which sprayed rooms had more half gravid to gravid specimens than unsprayed dwellings, though the proportion of catches for this species was low (4.2%, 30/721) for a meaningful analysis. A fairly high percentage (13%, 42/312) of fully fed *An. funestus* species was found exiting recently pyrethroid-treated structures in the two areas.
There was fairly low mortality in *Anopheles* mosquitoes collected from sprayed and unsprayed structures after a 24-hour holding period. The percentage of dead *An. funestus* and *An. gambiae s.l.* mosquitoes in the traps was significantly different between sprayed and unsprayed structures in both study sites (ANOVA: df = 2; \( F = 103.0; P = 0.002 \)), with the dead mosquitoes in sprayed structures constituting 16.7% (55/330), while unsprayed houses had 8.2% (32/391). The 24-hour survival rate in anopheline mosquitoes caught exiting sprayed and unsprayed structures was not significantly different (ANOVA: df = 3; \( F = 1.07; P = 0.48 \)), and the proportion of live mosquitoes after 24-hour holding period was 94.2% (259/275) for sprayed-house collections and 96.4% (346/359) for catches from unsprayed structures for both sites. *Anopheles funestus* demonstrated endophilic habits as indicated by the ratio of gravid to feed (gravid divided by fed) mosquitoes which was found to be constantly more than one.

Table 9: Percentage of exit window trap catches and 24-hour survival rates of *Anopheles* mosquitoes from lambda cyhalothrin sprayed and control structures in Burma Valley and Zindi

<table>
<thead>
<tr>
<th>Structure spray status</th>
<th>Mosquito species</th>
<th>N</th>
<th>Dead</th>
<th>Alive</th>
<th>Blood digestion stages</th>
<th>24 hour mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>UF</td>
<td>FF</td>
</tr>
<tr>
<td>Sprayed</td>
<td><em>An. funestus</em> group</td>
<td>312</td>
<td>16.3</td>
<td>83.7</td>
<td>9.0</td>
<td>13.4</td>
</tr>
<tr>
<td>Not sprayed</td>
<td><em>An. funestus</em> group</td>
<td>379</td>
<td>8.2</td>
<td>91.8</td>
<td>7.9</td>
<td>33.2</td>
</tr>
<tr>
<td>Sprayed</td>
<td><em>An. gambiae s.l.</em></td>
<td>18</td>
<td>27.8</td>
<td>72.2</td>
<td>11.1</td>
<td>0</td>
</tr>
<tr>
<td>Not sprayed</td>
<td><em>An. gambiae s.l.</em></td>
<td>12</td>
<td>8.3</td>
<td>91.7</td>
<td>0</td>
<td>16.7</td>
</tr>
</tbody>
</table>

UF: unfed, FF: fully fed, \( \frac{1}{2} \) G: half gravid, G: gravid

5.3.6 Density indices of *Anopheles* mosquitoes collected by pit shelter located at different distance from cattle kraals

One pit trap in each study site collapsed in March 2014, following heavy rains and could not be rehabilitated for use throughout the study period. A total of 101 *Anopheles* mosquitoes were collected, of which 98% (99/101) belonged to the *An. funestus* group and the remainder were *An. gambiae s.l.*. Of the *An. funestus* group caught, 54.5% (54/99) were from pit shelters located near (< 100 m) cattle kraals, while the remainder was collected in pits not adjacent to animal shelters in both sites. Outdoor daytime resting indices ranged from 0-1.72.
5.3.7 PCR species identification

The product of the PCR analysis showed that the 120 specimens taken randomly from morphologically identified *An. funestus* group collected by all four sampling methods (PSC, prokopac aspirator, PS and EWT) were all *An. funestus* s.s. However, all specimens morphologically identified as *An. gambiae* s.l. could not be identified to sibling species following unavailability of primers specific for this complex.

5.4 Discussion

Although *An. funestus* exhibited endophilic behaviour which was confirmed by EWTs with gravid to feed index of constantly more than one, the 16% exophilic habits shown by the results of this study is a cause for concern to the NMCP. This behaviour is of practical importance because it makes this proportion of vector less vulnerable to IRS, consequently reducing effectiveness of IRS as a strategy to combat malaria transmission. In Iran during 1960s, when dieldrin replaced DDT, *An. stephensi* species survived the high toxicity of the insecticide through exophilic habits in Zagros mountain areas (Hamon et al., 1970). Predominant endophilic mosquito populations may include varieties that exhibit exophilic habits. Probably, this tendency may be selected by the persistent use of insecticide or other human interventions, and it was considered the most likely reason for the failure of a WHO residual house spraying campaign in Garki district, Nigeria, to interrupt malaria transmission (Molineaux and Grammiccia, 1980).

In Zimbabwe, there has been little effort to assemble information on resting behaviour of malaria vectors, especially *An. funestus* species. The work by Masendu (1996) observed partial exophilic behaviour in *An. gambiae* s.l. in Gokwe and Binga Districts, Zimbabwe. Contrary, Dandalo (2007) reported major exophilic tendencies in *An. gambiae* s.l. and *An. merus* in Gokwe South District, Zimbabwe. Reports in Nigeria showed that *An. funestus* group and *An. gambiae* s.l. preferred to rest indoors (Oywole et al., 2007). In Tanzania, most *An. funestus* was caught indoors (Mahande et al., 2007), and these results are consistent with the findings of this work. Indoor residual spraying is likely to be effective only if the vector mosquito concerned is endophilic, because the mosquito needs to rest on the insecticide-treated surfaces for a sufficient time for it to pick a lethal dose (Pates and Curtis, 2005).
Of the endophilic *Anopheles* mosquitoes caught in this study, most were collected from the roofs/ceiling rather than the walls and other household goods. These results are slightly different to those observed in Suba District, Western Kenya, where the majority of mosquitoes were collected on the walls (Harbison *et al*., 2006). In Zimbabwe, it appears there are no documented studies on resting position preferences in mosquito populations of public health importance.

While indoor spraying of the walls and roofs/ceiling of houses with residual insecticides to reduce the longevity of indoor resting malaria vectors is crucial, for the past five years, NMCP in Zimbabwe has been only spraying the walls, leaving out most of the roofs/ceiling following non availability of spray extension lances to reach high resting positions. For mosquito species that rest indoors, it was generally thought that most males and females prefer to rest in the dark, low areas of the walls and therefore application of residual insecticides on the walls alone would reduce survival rates of indoor resting species to greatly reduce the chance of malaria transmission (Bidlingmayer, 1994; Pates and Curtis, 2005). The observations made in the present study, appear to suggest the urgent need for NMCP to procure spray extension lances, especially for Mutare and Mutasa Districts to facilitate spraying of high roofs/ceiling and other high sprayable locations not usually reachable without extension lance tubes.

The present work has shown that besides roofs and walls as resting locations indoors, it appears a small proportion of the *An. funestus* mosquitoes have the tendencies to rest on unsprayable locations such as furniture and other household objects. This selection of unsprayable locations for resting by *Anopheles* mosquitoes is of fundamental importance for vector control, suggesting that there might be a small proportion of persistence of malaria transmission despite high spray coverage in the study areas. The tendencies by relatively small proportion of vector mosquitoes to rest on unsprayable surfaces might be due to behavioural resistance induced by selection pressure of prolonged use of insecticides in the houses over the years.

Most wall-resting *An. funestus* mosquitoes were sampled from dark areas, less than 1 m from the floor level, followed by upper wall surfaces, more than 1.5 m above the ground level and close to the roof. Observations of this study are very similar to those documented in Puerto Rico (Clark *et al*., 1994), Panama (Perich *et al*., 2000), and Trinidad (Chadee, 2013). In sharp contrast, even though Harbison *et al*. (2006) collected a large number of mosquitoes resting at heights of less
than 0.8 m in Kenya, this number was not significantly different from the number resting above that height mark. In general, it is expected to find anopheline mosquitoes resting at low heights as these species prefer to be further away from the main indoor lights, all the openings, yet close enough to positions often occupied by people (Harbison et al., 2006).

Marevangepo (*personal communication*) reported WHO cone bioassay mortality of 34% in *An. gambiae* s.l. on sprayed walls at a height less than 0.5 m from the floor, while a wall height of more than 1 m of the same structure had 100% morality in Mutasa District, Zimbabwe. Although these WHO cone bioassay results were based on four sprayed structures only, they nonetheless suggest low deposits of insecticides on the lower heights, and these are the heights which were demonstrated by the present study as being the most preferred resting location for *An. funestus* mosquitoes. Elsewhere in Africa, work by Govere *et al.* (2001) evaluated quality of spraying using WHO cone bioassay in Mpumalanga Province, South Africa, and 100% mortality in *An. arabiensis* was observed in all cones placed on the bottom, middle and upper positions of each deltamethrin-treated wall, demonstrating an even deposition of insecticide on the sprayed surfaces.

Knowledge on the tendencies by mosquitoes to exit sprayed or unsprayed structures is of considerable importance in determining mosquito circulation from inside to outside and the degree of irritability as well as toxic effect on species populations leaving the treated houses (WHO, 1975). In Burma Valley and Zindi areas, where IRS is a major malaria intervention tool, this work collected a larger proportion of *An. funestus* populations in exit traps fixed on recently pyrethroid-treated structures, suggesting a possible pyrethroid resistance by this malaria vector.

Unfed females found in exit traps on sprayed houses were probably associated with insecticide irritancy or its excito-repellent property, while those collected from unsprayed structures were most likely denied feeding by host avoidance traits (WHO, 1975). A small fraction of fully blood-fed females observed from sprayed and unsprayed houses in this study may be those naturally exophilic mosquito species that leave the house soon after feeding (Pates and Curtis, 2005; Kulkami *et al.*, 2006; Fornadel and Norris, 2008).
High proportion of gravid mosquito collected in exit window traps in both sprayed and unsprayed structures confirmed the naturally endophilic behaviour of *An. funestus* (Pates and Curtis, 2005). Additionally, these gravid mosquito collections in recently pyrethroid-sprayed houses might further suggest possibility of insecticide resistance or poor spraying techniques which calls for further studies. The possibility of insecticide resistance or poor spraying techniques was demonstrated by high exit trap survival rates of *An. funestus* mosquitoes caught from recently pyrethroid-treated houses and kept over a 24-hour holding period.

Results of the present work agree with other studies carried out in Burkina Faso (Darriet, 1991), Mexico (Loyola *et al*., 1991) and Tanzania (Mnzava, 1995; Gerold, 1997), but totally in contrast with findings in Gokwe and Binga Districts, Zimbabwe, which reported more unfed *An. gambiae s.l.* mosquito collections in sprayed than unsprayed structures, with 100% species mortality regardless of spray status of the houses (Masendu, 1996). More recently Dandalo (2007) collected a larger proportion of *An. gambiae s.l.* mosquitoes in exit window traps in Gokwe South District, Zimbabwe, though data were not compared between sprayed and unsprayed houses.

This work, therefore, provides evidence for the need to maximize IRS benefit in the control of *An. funestus* mosquitoes by highlighting important preferred resting locations in Burma Valley and Zindi. This information supports the need for the NMCP to pay particular attention to provision of spray extension lances to reach high roofs, improve the training of spray operators to achieve even and adequate deposition of insecticide dosages on the surfaces and to conduct further studies to investigate insecticide resistance in the two study sites.
CHAPTER 6
BITING BEHAVIOUR OF *ANOPHELES FUNESTUS* POPULATIONS IN MUTARE AND MUTASA DISTRICTS: IMPLICATIONS FOR THE MALARIA CONTROL PROGRAMME

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3 This chapter was published as: Sande, S., Zimba, M., Chinwada, P., Masendu, H.T. and Makuwaza, A. 2016. Biting behaviour of *Anopheles funestus* populations in Mutare and Mutasa districts, Manicaland province, Zimbabwe: implications for the malaria control programme. *Journal of Vector Borne Diseases* **53**: 118-126.
6.1 Introduction

*Plasmodium falciparum*, *P. malariae* and *P. ovale* are the human malaria parasites in Zimbabwe, of which the first constitutes 95% of all causes of morbidity and mortality (Lukwa *et al.*, 2014). While *Anopheles arabiensis* is the principal vector of malaria parasites in Zimbabwe (Masendu *et al.*, 2005), *Anopheles funestus sensu stricto* is the primary vector in Mutare and Mutasa Districts (Sande *et al.*, 2015a). *Anopheles funestus* which prefers to breed in permanent or semi-permanent water bodies, generally exhibits patchy and discontinuous distribution patterns, and is highly anthropophilic, with major malaria episodes in sub-Saharan Africa (Gillies and De Meillon, 1968). This is more likely due to increased human-vector contact as a result of human settlements being usually located near to permanent or semi-permanent water bodies.

In Zimbabwe, indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) are the mainstays routinely applied to interrupt human-vector contact and malaria transmission. Mass and continuous distribution campaigns of LLINs have played an important role in reducing human-mosquito contact with consequent recent successes in malaria control (WHO, 2007b). Long-lasting insecticidal nets protect against mosquito bites that mostly occur indoors at night when people are sleeping. The host-feeding activities of mosquitoes have important implications for mostly humans, since it is by this behaviour that the transmission of malaria and other vector-borne disease-causing organisms take place. Studies on host preference, indoor and outdoor host-seeking behaviour as well as biting times of mosquitoes make an important contribution to determining environment and periods of malaria transmission risk, as well as form the basis for developing methods of personal protection against bites by vector mosquitoes.

Pyrethroid resistance in mosquito vector populations in most African countries is increasingly threatening the gains made by pyrethroid-treated LLINs (WHO, 2012). In Temotu Province, the Solomon Islands, *An. farauti* was reported to avoid insecticide exposure on the nets by shifting from feeding indoors (endophagic) to outdoors (exophagic) (Bugoro *et al.*, 2011). Evidence from recent studies in Africa suggest that malaria vector mosquitoes may avoid contact with insecticide imbedded in nets by biting predominantly outdoors or early evening and/or morning (Killeen *et al.*, 2006; Bugoro *et al.*, 2011). Russell *et al.* (2011) documented evidence of increased proportions of outdoor feeding among malaria vector populations following scaled up
insecticide-treated nets in rural Tanzania. This modification in mosquito behaviour that helps to avoid lethal effects of insecticide may result from the selection of genetically-inherited traits in response to increased coverage of LLINs and/or IRS (Pates and Curtis, 2005). Such inherited traits may render LLINs and/or IRS less effective in combating malaria transmission.

While Anopheles mosquito feeding behaviour has been studied extensively in the Afro-tropical region, host preferences, biting rhythms and infection rates of the An. funestus populations remain poorly understood in Zimbabwe. There have been few documented attempts to determine blood feeding venues and seasonality, biting periodicity, host preferences as well as accurate estimation of parasite infection in Zimbabwe. These are important entomological indicators which guide vector control strategies.

In Gokwe and Binga Districts, An. arabiensis was found to frequently feed on humans and animals indoors and outdoors, illustrating behaviours where some mosquitoes feed on any available blood source rather than looking for the preferred host (Masendu, 1996). The work by Dandalo (2007) on An. gambiae sensu lato (s.l.) and in Gokwe South District in showed the peak biting times to be from 21:00 to 22:00 hours. Changes in mosquito biting behaviour observed in Gokwe and Binga Districts (Masendu, 1996; Dandalo, 2007), and elsewhere in Africa (Killeen et al., 2006; Bugoro et al., 2011; Russell et al., 2011) might complicate studies on the determination of the feeding venues and times of vector mosquitoes as well as the ease with which LLINs can be implemented to combat malaria transmission. The behavioural characteristics of vector mosquitoes in malaria transmission may be different from region to region in Zimbabwe, and can well be understood only in the local context of available hosts, blood source preferences, behavioural resistance, and additional vector species.

Continuous monitoring and understanding behavioural responses of different vector mosquitoes to control tools is crucial to the National Malaria Control Programme (NMCP) as this facilitates the selection of the most effective control strategies. As Zimbabwe has joined the list of countries working towards eliminating malaria by 2030 (WHO, 2015a), the need to understand the biological implications of increased distribution of LLINs is of paramount importance. After the scaled up implementation of LLINs project by the NMCP in Mutare and Mutasa Districts, it is important to understand the behavioural responses of the major malaria vector, An. funestus, to
these tools. The aim of this study was to characterise the host-seeking behaviour of An. funestus by determining the human blood indices, host preferences, and sporozoite infection rate. The results presented here are the first study on host-seeking behaviour of the An. funestus populations in Zimbabwe.

6.2 Materials and methods

6.2.1 Study sites

Field work was conducted in the villages of Burma Valley (19°11’S, 32°48’E; elevation 679 m) and Zindi wards (18°22’S, 32°56’E; elevation 766 m) in Mutare and Mutasa Districts, respectively. Study sites details have been given in section 3.1 in the General Materials and Methods.

6.2.2 Mosquito sampling

Mosquitoes were sampled for one week each month over a period of one year (October 2013 to September 2014) using CDC, PSC, and PS (WHO, 2003). During each sampling week, light trap catches were conducted on Tuesday and Thursday nights, while PSC and PS collections were done on Wednesday and Friday mornings. Sampling by light traps was divided into two cohorts. In one cohort, five indoor and five outdoor light traps were set up in the evening and left overnight and trapped mosquitoes collected the following morning. In the other set, two light traps, one indoor and the other outdoor, were set up in the evening and the trapped mosquitoes were collected hourly till sunrise of the following morning.

For both indoor and outdoor trapping, the light trap was hung about 1.5 m above the ground close to the feet of an individual sleeping under an insecticide-free mosquito net. Two teams of two people working in six-hour relays aspirated trapped mosquitoes hourly throughout the night. In the two cohorts, light trapping was conducted from 18:00 to 06:00 hours for two nights per month at randomly-selected dwellings. The traps installed outdoors were set approximately 10-20 m away from houses. Catches were expressed as the mean proportion of mosquitoes collected per trap per night for overnight and mean proportion of mosquitoes per trap per hour for hourly collections. Indoor and outdoor temperature, relative humidity and rainfall patterns were
measured at hourly intervals using a digital thermometer, wet and dry bulb thermometer and rain gauge, respectively.

Centers for Disease Control light traps were used in this study instead of the standard human landing catch (HLC) method. The light traps were used as a proxy to approximately measure human biting rates (HBR) of vector mosquitoes since the method has a strong correlation to human landing catches, and three light traps would collect approximately equal numbers of vectors as two human collectors (Lines et al., 1991). In addition, it was shown that where use of vector control interventions is high and vector densities are low, CDC light traps can be used to monitor vector HBR, especially An. arabiensis, assuming that the mosquitoes that entered a trap during any hour, especially indoors, were those actively seeking a blood meal, and in most cases would bite human hosts in the same hour and area if the light trap was absent (WHO, 2003; Fornadel et al., 2010).

While the golden standard method for assessing HBR is the human landing catches, its extensive use has not been practical in many regions due to the increasing ethical and worker safety concerns that this mosquito sampling method increases the risk of exposure of collectors to infectious mosquitoes (Fornadel et al., 2010). In addition, the method appears to collect non-standardized data because of variability of the attractiveness and skill of collectors (Fornadel et al., 2010).

Pyrethrum spray collection and PS methods were included mainly to determine the human blood index (HBI) and sporozoite rate. Indoor-resting adult mosquitoes were collected by the PSC method (WHO, 2003) in 20 conveniently sampled bedrooms. On each study day, mosquitoes were collected between 06:00 to 10:00 hours from each selected bedroom. Outdoor-resting adult mosquitoes were collected using 20 pit shelters, 10 for each site, conveniently dug in the villages of Burma Valley and Zindi. Pits were separated from each other by a distance of not less than 200 m and located in tree-shaded environments. Each pit shelter was 1.8 m deep, 1.5 m long and 1 m wide, with four small horizontal cavities of 0.3 m deep dug on the walls at a height of 0.6 m from the bottom. Mosquitoes were aspirated from each PS for two days in each month for the study period.
Female anophelines from all collections were counted, their abdominal status determined (unfed, partially fed, fully fed or gravid) and identified using morphological characters (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Mosquitoes belonging to the An. funestus group and An. gambiae s.l. were placed singly in labelled eppendorf tubes containing silica gel and transported to National Institute of Health Research (NIHR) laboratory in Harare for further processing.

6.2.3 PCR species identification

Polymerase chain reaction identification of members of the An. funestus group was determined using DNA extracted from two legs or wings of each morphologically-identified specimen following the method described by Koekemoer et al. (2002). The PCR assays were to confirm that the assays on blood meal sources and sporozoite rates were conducted only on An. funestus s.s. sibling species. Polymerase chain reaction species identification was conducted only to those specimens tested for blood meal sources and sporozoites.

6.2.4 Blood meal sources and human blood index

All blood-fed An. funestus mosquitoes collected were assayed using cytochrome b-based multiplex PCR for blood meal source identification (Kent and Norris, 2005). Abdomens of fully-fed specimens were separated from the heads and thoraces, ground in 50 μL phosphate-buffered saline and analysed for blood meal sources for human, bovine, dog, goat, and pig primers. Human blood index was calculated by dividing the number of An. funestus mosquitoes with human blood by the total number of An. funestus engorged with blood. Mixed blood meal sources were each treated as two separate blood meal sources.

6.2.5 Sporozoite detection in infected mosquitoes and entomological inoculation rate

Heads and thoraces were removed from the abdomens of dried mosquitoes and tested by enzyme-linked immunosorbent assay (ELISA) for the circumsporozoite (CS) protein antigen in the salivary glands using monoclonal antibodies specific for P. falciparum following the standard protocol described by Wirtz et al. (1987). Tests were conducted to detect only Plasmodium falciparum because it is the predominant malaria parasite species in Zimbabwe,
constituting 95% of all cases (Lukwa et al., 2014). Mosquitoes were pooled and tested in groups of less than 10 according to collection method and date (Galardo et al., 2007; Magris et al., 2007). Enzyme-linked immunosorbent assay positive samples from the initial screening were re-tested to confirm positives and to quantify the amount of CS protein for each sample. Sporozoite rate was obtained by dividing the number of An. funestus which contained P. falciparum sporozoites with the total number of An. funestus tested (WHO, 2003).

6.2.6 Data analysis

The indoor and outdoor human biting densities and biting times, blood meal sources and differences in sporozoite rates in Anopheles mosquitoes collected in the two study sites for the whole sampling period were compared using the one-way ANOVA test at 0.05 level of significance.

6.3 Results

6.3.1 Anopheline mosquito composition and abundance

Overall, 2,268 adult female Anopheles mosquitoes were collected by CDC light traps, PSC and PS methods (Table 10). All the specimens collected by the three methods fell into two major groups of Anopheles mosquitoes: the An. funestus group (98.3%) and An. gambiae s.l. (1.7%). Of the entire Anopheles mosquitoes collected, the majority were caught by CDC light traps, followed by PSC, and the least by PS. There were no significant differences in the number of Anopheles mosquitoes collected at the two study sites (ANOVA: df = 4; F = 2.87; P = 0.06). All the CDC mosquito collections in both sites were unfed. The PSC method had the highest percentage of fully-fed (96.0%) and gravid (98.5%) anopheline mosquitoes.

6.3.2 Indoor and outdoor catches of Anopheles mosquitoes

A total of 1,096 anopheline mosquitoes were collected by CDC light traps set indoors and outdoors overnight for the entire one year study period at the two sites (Table 11). The An. funestus group constituted the majority of the total anopheline mosquitoes collected. Of these, 68.8% were sampled indoors and the remainder was caught outdoors. The indoor and outdoor flight densities for the An. funestus group and An. gambiae s.l. were significantly different.
Comparison of flight activities for *An. funestus* between sites revealed no significant differences in mosquito numbers both indoors (ANOVA: df = 1; \( F = 0.02; P = 0.89 \)) and outdoors (ANOVA: df = 1; \( F = 0.01; P = 0.90 \)).

**Table 10:** Percentage *Anopheles* mosquito species composition by sampling method as identified by morphological means

<table>
<thead>
<tr>
<th>Site</th>
<th><em>Anopheles</em> species</th>
<th>N</th>
<th>CDC</th>
<th>PSC</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>UF</td>
<td>FF</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>UF</td>
<td>FF</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>UF</td>
<td>FF</td>
<td>G</td>
</tr>
<tr>
<td>Burma Valley</td>
<td><em>An. funestus s.l.</em></td>
<td>1,139</td>
<td>69.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>An. gambiae s.l.</em></td>
<td>21</td>
<td>71.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Zindi</td>
<td><em>An. funestus s.l.</em></td>
<td>1,090</td>
<td>63.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>An. gambiae s.l.</em></td>
<td>18</td>
<td>61.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>2,268</td>
<td>66.1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 11:** Percentage variation of indoor and outdoor catches of *Anopheles* mosquitoes

<table>
<thead>
<tr>
<th>Site</th>
<th>N</th>
<th><em>An. funestus</em> group</th>
<th>N</th>
<th><em>An. gambiae</em> complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Indoor</td>
<td>Outdoor</td>
<td></td>
</tr>
<tr>
<td>Burma Valley</td>
<td>588</td>
<td>68.5</td>
<td>31.5</td>
<td>13</td>
</tr>
<tr>
<td>Zindi</td>
<td>488</td>
<td>69.1</td>
<td>30.9</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>1,076</td>
<td>68.8</td>
<td>31.2</td>
<td>20</td>
</tr>
</tbody>
</table>

### 6.3.3 Seasonal occurrence of anopheline mosquitoes

The indoor and outdoor CDC light catches of the *An. funestus* group and *An. gambiae s.l.* varied according to the season of the year (Table 12), with approximately light trap collection densities of 3.3 and 2.0 mosquitoes per trap per night during the wet season (November to March) for *An. funestus* mosquitoes in Burma Valley and Zindi, respectively. Most of the *An. funestus* group and *An. gambiae s.l.* mosquitoes were collected during the wet season at both sites. *Anopheles funestus* indoor catches during the dry season (April to October) was lower than wet season. In general, the occurrence of *An. funestus* populations persisted from late wet to early dry season and completely absent in the mid-dry season which coincided with winter. Comparison of seasonal flight activity for *An. funestus* populations and *An. gambiae s.l.* revealed significant
differences (ANOVA: df = 4; $F = 8.65; P = 0.00$) in mosquito densities sampled indoors and outdoors between seasons (wet and dry).

### 6.3.4 Flight rhythm of the Anopheles funestus group in Burma Valley and Zindi

In both study sites, the majority of adult female *An. funestus* collected indoors and outdoors exhibited flight activity almost throughout the night (Figure 2). Indoor and outdoor mosquito flight activity commenced at 20:00 hours, but steadily increased up to 23:00 hours, and decreased either gradually or sharply thereafter to the point of almost zero flight around 01:00 hours. However, flight activity peaks varied slightly at collection sites, with two peaks: the first peak during the first six hours of the night and the second during the last six hours. From 02:00 hours, the indoor flight activity rhythm increased steadily with peak being observed between 02:00 and 03:00 hours in Burma Valley and 02:00 to 04:00 hours in Zindi. The major flight activity periodicity occurred during the second half of the night. The observed indoor flight activity of the *An. funestus* group exceeded the outdoor flight activity in both sites and there were significant differences between the indoor and outdoor flight activity (ANOVA: df = 3; $F = 8.25; P < 0.01$). The outdoor flight activities of the *An. funestus* group were apparently similar in both areas, except for a sharp fall after 03:00 hours in Burma Valley.
Table 12: Seasonal flight densities of indoor and outdoor CDC light trap anopheline collections from Burma Valley and Zindi

| Site       | Species       | Period               | Indoor |  | Outdoor |  |
|------------|---------------|----------------------|--------|  |---------|---|
| Burma Valley | *An. funestus* | Wet season (Nov-Mar) | 25     | 4.82 ± 0.65 | 3.31-6.63 | 25 | 1.80 ± 0.22 | 1.12-2.43 |
| Burma Valley | *An. funestus* | Dry season (Apr-Oct) | 24     | 2.43 ± 0.88 | 0-5.92    | 23 | 1.36 ± 0.66 | 0-4.71    |
| Burma Valley | *An. gambiae s.l.* | Wet season (Nov-Mar) | 25     | 0.16 ± 0.12 | 0-0.53    | 25 | 0.10 ± 0.05 | 0-0.20    |
| Burma Valley | *An. gambiae s.l.* | Dry season (Apr-Oct) | 24     | 0.09 ± 0.07 | 0-0.50    | 23 | 0.03 ± 0.03 | 0-0.23    |
| Zindi      | *An. funestus* | Wet season (Nov-Mar) | 23     | 3.84 ± 0.45 | 2.93-5.33 | 22 | 1.86 ± 0.33 | 1.01-2.73 |
| Zindi      | *An. funestus* | Dry season (Apr-Oct) | 24     | 1.10 ± 0.82 | 0-1.82    | 23 | 0.38 ± 0.35 | 0-1.52    |
| Zindi      | *An. gambiae s.l.* | Wet season (Nov-Mar) | 23     | 0.52 ± 0.23 | 0-1.00    | 22 | 0.10 ± 0.06 | 0-0.31    |
| Zindi      | *An. gambiae s.l.* | Dry season (Apr-Oct) | 24     | 0.46 ± 0.27 | 0-1.91    | 23 | 0.08 ± 0.04 | 0-0.32    |
6.3.5 PCR analysis of the Anopheles funestus sibling species

A total of 726 An. funestus group mosquitoes which were either fully blood-fed or gravid were PCR-assayed for identification to sibling species. The analysis revealed that 91.4% were An. funestus.s.s., 4.9% An. leesoni, and 3.7% could not amplify, despite the successful amplification of positive control. However, fully blood-fed and gravid An. gambiae s.l. specimens were not PCR-tested to sibling species due to non-availability of primers to differentiate members of the An. gambiae complex.

6.3.6 Identification of blood meal sources of Anopheles funestus

The PCR diagnostic identified 272 blood meals of humans and domestic animals from engorged An. funestus collected from the two study sites (Plate 15). The results indicated that the majority of An. funestus fed on human blood source (anthropophily), resulting in HBI of about 64% (n = 175) for both indoor and outdoor resting mosquitoes collected at the two sites (Table 13). The remaining proportions of blood meals were received from domestic animals (zoophily). Among
the animals, *An. funestus* showed fairly high preference for bovine blood, with dog blood least preferred. Only one specimen showed mixed bovine and goat blood sources.

![Image of PCR gel](image)

Plate 15: PCR identified blood meal sources of *Anopheles funestus* collected from Burma Valley and Zindi areas. Lanes I and 13: 100bp molecular ladder, Lane 12: Negative control, lane 11: Positive control human, lane 2: Pig (453 base pair), lanes 3, 7, 8, 9 and 10: Human (334 base pair), lane 4: Mixed (Goat 132 and Bovine 561 base pair), lane 6: Bovine (561 base pair), lane 5: Dog (680 base pair)

Table 13: Percentage of blood meal sources of *Anopheles funestus* collected from Burma Valley and Zindi

<table>
<thead>
<tr>
<th>Site</th>
<th>Blood meal source</th>
<th>N</th>
<th>Human</th>
<th>Bovine</th>
<th>Dog</th>
<th>Goat</th>
<th>Pig</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burma Valley</td>
<td></td>
<td>124</td>
<td>63.7</td>
<td>13.7</td>
<td>1.6</td>
<td>16.1</td>
<td>4.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Zindi</td>
<td></td>
<td>148</td>
<td>64.8</td>
<td>14.9</td>
<td>1.4</td>
<td>11.5</td>
<td>7.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>272</td>
<td>64.2</td>
<td>14.3</td>
<td>1.5</td>
<td>13.8</td>
<td>5.8</td>
<td>0.4</td>
</tr>
</tbody>
</table>

6.3.7 Detection of circumsporozoite antigen and entomological inoculation rate

Table 14 shows the total number of *An. funestus* specimens tested for *P. falciparum* circumsporozoite and infection rates using ELISA. Only about 2% (n = 8) of the specimens
tested positive for *P. falciparum* sporozoites for the mosquitoes collected from the two study sites. Partitioning the infection rates by site indicated a close to 2% sporozoite rate for Burma Valley and about 1% for Zindi. However, the number of infected mosquitoes between the two study sites was significantly different (*P* > 0.05).

Table 14: Number of *Anopheles funestus* tested and percentage found with *Plasmodium falciparum* circumsporozite antigens

<table>
<thead>
<tr>
<th>Site</th>
<th>No. tested</th>
<th>Sporozoite rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burma Valley</td>
<td>211</td>
<td>2.4</td>
</tr>
<tr>
<td>Zindi</td>
<td>243</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>454</strong></td>
<td><strong>1.8</strong></td>
</tr>
</tbody>
</table>

### 6.4 Discussion

Work by Lines *et al.* (1991) in Tanzania and Fornadel *et al.* (2010) in Zambia demonstrated that CDC light trap is comparable to human landing catches and therefore considered a proxy tool for sampling malaria vector mosquitoes that would otherwise feed on humans. While the light trap has been shown to underestimate the actual mosquito biting risk (Mbogo *et al*., 1993), CDC traps are important as they can be used to monitor HBRs in malaria vector mosquitoes in areas where use of vector control interventions is high, with low vector densities (Fornadel *et al*., 2010). Mosquitoes caught by CDC light traps in the present work compares with those reported in similar studies (Lines *et al*., 1991; Davis *et al*., 1995) in that each trap was set next to a human and that the majority of the mosquitoes collected were unfed, suggesting that the mosquitoes were caught in the act of host-seeking.

Investigating host-seeking behaviour, host preferences and presence of sporozoites in *Anopheles* mosquitoes is necessary to understanding their probability as vectors of malaria. Sharp (1983) demonstrated that the biting behaviour of *Anopheles* mosquitoes can be markedly disrupted by changes in environmental factors during the night, especially rain and wind. Wind is known to have a direct effect on mosquito flight (Snow, 1980; Gillies and Wilkes, 1981). However, no major adverse weather conditions were encountered during the entire period of the study and it
was possible to collect mosquitoes at different venues and time, which indicated important entomological information that could be utilized to implementing appropriate vector control interventions.

In Burma Valley and Zindi, An. funestus was found to be the major anopheline in the two areas and the species demonstrated predominantly indoor flight patterns. An estimate of the degree of endophagy and exophagy can be obtained when the relative proportions of the mosquitoes attempting to bite indoors and outdoors are compared (Krafsur, 1977). The CDC light catches in this study demonstrated that mosquitoes were more abundantly indoors (68%) than outdoors (32%), suggesting that the indoor nocturnal host-seeking tendencies of An. funestus and An. gambiae s.l. could be interrupted by the intra-domiciliary use of LLINs by the majority of residents of Burma Valley and Zindi areas. However, the relevance of outdoor host-seeking behaviour of mosquitoes to vector control might depend hugely on the coincidence between outdoor biting intensity and human outdoor activity (Kabbale et al., 2013).

Comparative historical indoor and outdoor biting profiles for malaria vector mosquitoes in Zimbabwe are lacking from published literature. However, the results of the present study are consistent with the previous studies in Uganda in which most An. funestus populations and An. gambiae s.l. fed indoors (Kabbale et al., 2013). The finding of this work contradicts those from other studies which showed outdoor host-seeking profiles in An. arabiensis from Nigeria (Oyewole et al., 2007), and An. gambiae s.s. from Bioko Island, Equatorial Guinea (Reddy et al., 2011). In other example, An. neivai in Colombian Pacific, fed outdoors following exposure to insecticide pressure (Escovar et al., 2013). Although this study showed dominance in indoor CDC light trap catches, the densities appear to be strongly dependent upon seasons (wet or dry).

In the present work, seasonal mosquito flight profiles were described and categorised into wet and dry seasons. The densities of An. funestus populations and An. gambiae s.l. were higher during the wet season than dry season which, in Zimbabwe, corresponds with the period of the year, usually February/March when vector density is generally at its peak following abundance of breeding sites, suitable temperatures and relative humidity (Masendu, 1996).
Little is known about the seasonal host-seeking behaviour of malaria vectors in Zimbabwe for comparative purposes. However, similar results have been reported in Nigeria for An. gambiae but with An. funestus having high dry seasonal biting tendencies (Oyewole et al., 2007). The results of the current study suggest that while it is important to conduct mass and continuous net distribution campaigns all year round, it is critical to intensify net hang-up campaigns in wet season, but this should not preclude this activity the rest of the year.

When the indoor and outdoor components of hourly trap catches of An. funestus populations were examined, it was noted that the rhythm of flight activity fluctuated in a similar fashion throughout the night in both sites. Knowledge on the biting times of anopheline mosquitoes is crucial in ascertaining whether peak biting period coincides with that part of the night after the inhabitants retired to bed. An important finding in this context was the general nocturnal mosquito flight cycles.

The first flight activity peak which occurred prior to midnight suggests the possibility of continued malaria transmission despite net ownership and use as this was a period when probably a fairly small proportion of the rural population might still be out of bed. Further, the second peak was observed towards dawn, a period which might put some people at risk of mosquito bites as they might be out of bed for early morning household chores. This suggests that the use of mosquito repellents would be effective to complement LLINs during the double peaks when some people would not be bed under LLINs.

Although Moiroux et al. (2012) also observed two peaks of biting activity of An. funestus, the times of the first and second peaks which were recorded between 12-midnight and 01:00 hours, and 03:00 and 04:00 hours, respectively, differed from the peak times of this study. In contrast, in Masakadza area, Zimbabwe, Dandalo (2007) reported that biting of An. gambiae complex mosquitoes commenced at 19:00 hours and ceased at 05:00 hours with biting peaks at 22:00 hours. In Kenya, the biting of An. gambiae s.l. gradually increased throughout the night with a peak three hours before dawn (Braack et al., 1994). However, the explanations for two peaks and the sharp fall in CDC light trap catches between midnight and 01:00 hours were unclear and could not be established from the present work.
The risk of transmission of mosquito-borne diseases to human populations depends greatly on the degree of human biting by vector mosquitoes, which in turn would be influenced by abundance, distribution, and host blood source preferences. In relation to the host blood meal sources, the degree of risk would depend largely on the anthropophilic or zoophilic profile of the vector mosquito. More than 64% of the blood meals identified from *An. funestus* collected from Burma Valley and Zindi were obtained from human host, suggesting that this population of *An. funestus* is highly anthropophilic, and this tendency to feed on human blood increases vectorial capacity (Animut *et al.*, 2013). The high HBI in the present study might be attributed to the attraction of the species to human habitats where no domestic animals are kept. Human blood index in *An. funestus* species observed in this study is consistent with maintaining high levels of malaria transmission in the almost total absence of other vector species and is an important factor in the epidemiology of the disease as well as in estimating human-vector contact for determining malaria transmission intensity and planning for its control. However, large proportion of mixed human and animal blood meals widely reported in other studies in *An. funestus* mosquitoes (Adugna *et al.*, 1996; Habtewold *et al.*, 2001; Kent *et al.*, 2007), and *An. arabiensis* (Tirados *et al.*, 2006; Animut *et al.*, 2013) were not observed in the present work. However, this sharp contrast could not be clearly understood.

The sporozoite rate of about 2% in *An. funestus* in Burma Valley and Zindi specimens was low. Annual *P. falciparum* infectious bites lower than 10% sporozoite rate indicate its unstable transmission intensity (Okello *et al.*, 2006) and risk of epidemics (Lindsay *et al.*, 1998). Basing on this submission, the results from the current study suggest that malaria transmission in Burma Valley and Zindi might be unstable with possibility of spontaneous epidemics which calls for vigilant surveillance to avert unforeseeable disasters.

In Burma Valley and Zindi areas, malaria control strategies have greatly targeted intradomiciliary vector mosquitoes largely through the provision of LLINs with net ownership of about 100% apiece. This tool has been proven effective against epidemiologically important anopheline vectors targeting prominently indoor biting behaviour. However, where human biting occurs outdoors and/or before midnight and/or towards dawn when people are not protected by LLINs, indoor-based mosquito net intervention might not be sufficient to reduce malaria incidence to a point where it is no longer a public health problem. An important finding in this
context was that generally, the nocturnal mosquito flight cycles commenced at 20:00 hours, with double peaks between 22:00 and 23:00 hours during the first six hours of the night, and between 02:00 and 04:00 hours for the second six hours. By 06:00 hours, flight activities would have almost completely ceased. As such, it is clear from the results of this study that consistent use of nets every night all year round, use of personal protective clothing and repellents during peak mosquito densities might suppress malaria transmission. More so, the biting patterns of the *An. funestus* populations warrant further study.
CHAPTER 7

THE EMERGENCE OF INSECTICIDE RESISTANCE IN THE MAJOR MALARIA VECTOR ANOPHELES FUNESTUS (DIPTERA: CULICIDAE) FROM SENTINEL SITES IN MUTARE AND MUTASA DISTRICTS

4 This chapter was published as: Sande, S., Zimba, M., Chinwada, P., Masendu, H.T., Mazando, S. and Makuwaza, A. 2015. The emergence of insecticide resistance in the major malaria vector Anopheles funestus (Diptera: Culicidae) from sentinel sites in Mutare and Mutasa districts, Zimbabwe. Malaria Journal 14:466.
7.1 Introduction

Human malaria remains one of the most important public health challenges worldwide. In 2013, there were an estimated 198 million episodes of malaria and about 584,000 deaths globally (WHO, 2014a). Among the malaria-endemic countries in sub-Saharan Africa, malaria contributed 20-30% of the outpatient attendance in Zimbabwe, with about 1.5 million cases occurring annually over the past five years (Choto et al., 2010). Approximately 98% of the cases are caused by Plasmodium falciparum transmitted primarily by Anopheles arabiensis, with Anopheles gambiae sensu stricto and Anopheles funestus sensu stricto, the secondary vectors in most regions of the country. Choi et al. (2014) and Sande et al. (2015a) have reported An. funestus as the major vector of malaria in Mutare and Mutasa Districts of Manicaland Province in Zimbabwe.

Improved diagnostic testing and a wider availability of effective medicines to treat malaria, as well as to control vectors predominantly through the use of indoor residual spraying (IRS) and long lasting insecticidal nets (LLINs), are the global key interventions for interruption of malaria transmission (WHO, 2014b). Several studies have shown the efficacy of IRS and LLINs in reducing malaria incidence in almost all settings (Lengeler, 2004; Pluess et al., 2010).

Malaria control in Zimbabwe relies heavily on IRS and LLINs to target endophilic and endophagic vector mosquitoes, respectively. Presently, IRS and LLINs depend on the four most common, WHO-recommended, classes of insecticides: organochlorines, organophosphates, pyrethroids, and carbamates. Of these, pyrethroids account for the majority of IRS coverage worldwide and are at the moment used in treatment of all LLINs (WHO, 2013a).

Since the 1940s, residual spraying with DDT and more recently pyrethroids has been National Malaria Control Programme’s (NMCP) dominant/primary vector control practice in Zimbabwe. Mosquito nets traditionally played a much smaller role until the introduction of LLIN campaigns under the universal coverage goal over the past few years. When the LLIN distribution campaign began, there was no clear rationale for the balance of LLINs and IRS coverage in Zimbabwe as guided by WHO (2014b) recommendations. The high reliance on insecticide-based malaria control in public health, agriculture and at household levels has increased the selection pressure...
exerted by insecticides on malaria vectors (WHO, 2012). The emergence and spread of insecticide resistance among malaria vectors has placed global control efforts at high risk.

Insecticide resistance is the ability of an insect population to survive exposure to the dosage of a given compound that is lethal to the majority of individuals of a susceptible lineage of the same species (WHO, 2012). Malaria vectors are able to resist the action of insecticides due to various resistance mechanisms. Among these mechanisms: metabolic resistance, which occurs when endogenous, insecticide-detoxifying enzymes become more efficient in metabolizing the insecticide, preventing it from reaching its target in the nervous system, and target site resistance, which results from modification on the site of action in resistant strains of vectors, such that the insecticide no longer binds effectively, are the most important, although metabolic resistance is the most common (Brogdon and McAllister, 1998).

Pyrethroid resistance, conferred by reduced target site sensitivity arising from a single point mutation in the sodium channel gene, at times referred to as knockdown resistance, has been confirmed in *An. gambiae s.s.* in West, Central and East Arica (IRAC, 2011). A study by Hunt et al. (2010) documented insecticide resistance to permethrin, deltamethrin, bendiocarb, and propoxur in *An. funestus* populations collected in Likoma Island in Lake Malawi. Chanda et al. (2011) reported DDT, lambda-cyhalothrin and deltamethrin resistance in *An. funestus* and *An. gambiae s.s.* collected in Zambia. Anopheles funestus collected in Mozambique and Uganda showed resistance to bendiocarb, permethrin, deltamethrin, and lambda-cyhalothrin (Morgan et al., 2010; Abilio et al., 2011). In Kwazulu/Natal, South Africa, *An. funestus* was found to be resistant to both pyrethroids and carbamates (Hargreaves et al., 2000).

Despite the long history of IRS in Zimbabwe, there have been few instances when resistance has been recorded (Lukwa et al., 2014). *Anopheles arabiensis* resistance to benzene hexachloride was recorded in Chiredzi District (Green, 1982), one relating to DDT in Gokwe (Masendu et al., 2005), and more recently pyrethroid resistance in Gokwe (Munhenga et al., 2008). However, there are no major published studies on insecticide resistance in *An. funestus* in Zimbabwe. The first *An. funestus* resistance to deltamethrin, lambda-cyhalothrin and bendiocarb was reported by Choi et al. (2014) in Mandeya ward, Mutasa District.
The lack of data on the status of insecticide resistance in *An. funestus*, the presence of this vector in recently pyrethroid-sprayed houses in villages around Burma Valley, Mutare District, Zimbabwe, and nearby Zindi area in Mutasa District, and high dependency on pyrethroid-based IRS and LLINs, is a cause for concern to the Zimbabwe NMCP. This study was aimed at assessing insecticide resistance in *An. funestus* populations from Burma Valley and Zindi areas in Mutare and Mutasa Districts, respectively.

7.2 Methods

7.2.1 Study sites

The study was conducted in Mutare and Mutasa Districts in Manicaland Province, located east of Zimbabwe, 263 and 270 km, respectively, from Harare, and bordered to the east by Manica Province in Mozambique. Detailed description of the study sites have been given in section 3.1 in the General Materials and Methods.

7.2.2 Collection of *Anopheles funestus* populations

Indoor-resting adult mosquitoes were collected from houses between 06:00 and 10:00 hours using a prokopac battery-powered aspirator (Vazquez-Prokopec *et al.*, 2009). Live mosquitoes were identified to species level using morphological features (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Mosquitoes identified as belonging to the *An. funestus* group were divided into two cohorts and held in cages where they were fed with 10% sugar solution. One cohort was used immediately for WHO insecticide susceptibility bioassays and the other batch transferred to NIHR insectary in Harare to allow for oviposition.

7.2.3 Laboratory processing of mosquitoes

Live blood-fed and gravid adult female *An. funestus* were pooled and individually isolated and allowed to lay eggs. Larvae were reared through to F1 adults under standard insectary conditions of 25-27°C and 70-80% relative humidity. Polymerase chain reaction (PCR) using two legs per mosquito was carried out following the protocol of Koekemoer (Koekemoer *et al.*, 2002) to
confirm the sibling species of all females that laid eggs, and susceptibility and intensity resistance assays were conducted on the F1 progeny only of *An. funestus s.s.* females.

### 7.2.4 Insecticide susceptibility tests

Randomly selected, non-blood fed F1 progeny (3-5 days old) and gravid wild caught samples were subjected to standard WHO susceptibility tests (WHO, 2013a). Standard insecticide-treated papers supplied by WHO (Malaysia) were used to test for susceptibility to 4% DDT, 0.05% deltamethrin, 0.05% lambda-cyhalothrin, 0.5% etofenprox, 0.1% bendiocarb, and 1% pirimiphos methyl. Twenty to 25 female mosquitoes were exposed in each tube. Negative controls consisted of untreated papers, impregnated with different oil according to the insecticide used. Knockdowns were recorded 10, 15, 20, 30, 40 min through to one hour after the start of exposure. Final mortality was scored 24 hours post exposure and a 10% sugar solution was provided to survivors. Where the mortality in the control group was above 5% but less than 20%, correction of mortality was made by applying Abbott’s formula, with the test results discarded when control mortality was more than 20%. Results were accepted if no mortality was observed in the control. The WHO (2013a) criterion for interpretation of results was followed for considering vector species susceptible (mortality 98-100%), potentially resistant (mortality 90-97%) and resistant (mortality <90%).

### 7.2.5 Resistance intensity assays

F1 progeny female mosquitoes were exposed to 0.05% lambda-cyhalothrin, 0.05% deltamethrin, 0.5% etofenprox, and 0.1% bendiocarb-treated papers continuously for eight hours with knockdown being recorded at 5, 10, 15, 20, 30, 40, 50, 60, 80, and 120-min intervals, and hourly thereafter up to eight hours. The eight-hour cut-off was purposely selected as the likely time a mosquito might come into contact with a sprayed wall/surface before or after a taking blood meal (Choi *et al.*, 2014).

### 7.2.6 Data analysis

WHO (2013) guideline for evaluating susceptibility in mosquito populations was followed in which mortality of 98-100% indicates susceptibility, 90-97% suggests potential resistance that
needs to be confirmed, and less than 90% indicates resistance. Data for the two study sites were tested using two-factor without replication Analysis of Variance (ANOVA), at 5% level of significance.

7.3 Results

7.3.1 Mosquito collections

A total of 846 Anopheles mosquitoes were collected resting inside recent pyrethroid-sprayed houses in the villages surrounding Burma Valley and Zindi over a two-week period in February 2014. Eight-hundred and thirty-six were identified morphologically as belonging to the An. funestus group, seven to the An. gambiae s.l. and the remaining three to other Anopheles species. Of the An. funestus group, 390 live mosquitoes were transported to NIHR for oviposition and PCR-based species identification, while 446 wild An. funestus female of unknown age were tested for insecticide resistance at the field insectaries with no temperature and relative humidity control. The results of these tests are summarized in Table 15. The wild-caught An. funestus group showed evidence of pyrethroid and carbamate resistance, but were susceptible to DDT and organophosphates. However, the sample size of wild An. gambiae s.l. females was too small (n = 7) to conduct meaningful susceptibility/resistance tests.

7.3.2 Mosquito rearing and PCR-species identification

From the 390 samples transported to NIHR insectary, 220 oviposition Eppendorf tubes were set up with individual gravid An. funestus females. About 134 batches were obtained, and more than 1,900 F1 adult mosquitoes emerged from both sites. The results from the PCR-based assays confirmed that all the 220 females that laid eggs, as well as the 446 wild adults used in the susceptibility/resistance test, were An. funestus s.s., while analysis of wild An. gambiae s.l. showed that An. arabiensis was predominant (71.4%, 5/7) followed by a non-malaria vector, An. quadriannulatus (28.6%, 2/7).
Table 15: Percentage 24 h post-exposure mortality observed in WHO susceptibility tests carried out on wild caught members of the *Anopheles funestus* group in Burma Valley and Zindi

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Site</th>
<th>n</th>
<th>mortality (%)</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05% Lambda-cyhalothrin</td>
<td>Burma Valley</td>
<td>47</td>
<td>6.5</td>
<td>R</td>
</tr>
<tr>
<td>(pyrethroid)</td>
<td>Zindi</td>
<td>20</td>
<td>0</td>
<td>R</td>
</tr>
<tr>
<td><em>P</em>-value (between sites)</td>
<td></td>
<td>-</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>0.05% Deltamethrin</td>
<td>Burma Valley</td>
<td>31</td>
<td>12.9</td>
<td>R</td>
</tr>
<tr>
<td>(pyrethroid)</td>
<td>Zindi</td>
<td>*ND</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><em>P</em>-value (between sites)</td>
<td></td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>0.5% Etofenprox (pseudopyrethroid)</td>
<td>Burma Valley</td>
<td>33</td>
<td>3.0</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>39</td>
<td>15.4</td>
<td>R</td>
</tr>
<tr>
<td><em>P</em>-value (between sites)</td>
<td></td>
<td>-</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>0.1% Bendiocarb (carbamate)</td>
<td>Burma Valley</td>
<td>38</td>
<td>21.1</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>43</td>
<td>2.3</td>
<td>R</td>
</tr>
<tr>
<td><em>P</em>-value (between sites)</td>
<td></td>
<td>-</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>4% DDT (organochlorine)</td>
<td>Burma Valley</td>
<td>36</td>
<td>100</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>30</td>
<td>100</td>
<td>S</td>
</tr>
<tr>
<td><em>P</em>-value (between sites)</td>
<td></td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>1% Pirimiphos-methyl (organophosphate)</td>
<td>Burma Valley</td>
<td>30</td>
<td>100</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>34</td>
<td>100</td>
<td>S</td>
</tr>
<tr>
<td><em>P</em>-value (between sites)</td>
<td></td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>Burma Valley</td>
<td>35</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>30</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><em>P</em>-value (between sites)</td>
<td></td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

*ND, not done

### 7.3.3 Insecticide susceptibility assays

Table 16 presents the mean mortalities and the standard error of *An. funestus* F1 progeny females that originated from the villages in Burma Valley and Zindi following exposure to insecticide-treated papers. Mortality in unexposed controls from both sites was less than 5% in all experiments and no correction of test sample mortality data was therefore required. The treated papers used were assayed on a susceptible laboratory strain of *An. arabiensis* and showed 100% mortality for all specimens and replicates. *Anopheles funestus* was resistant to lambda-cyhalothrin, deltamethrin and etofenprox (pyrethroids), and bendiocarb (carbamate), but susceptible to DDT (organochlorine) and pirimiphos-methyl (organophosphate) at both
collecting sites. There were no significant differences in mortality of mosquitoes from Burma Valley and Zindi after exposure to pyrethroids (ANOVA: df = 4; $F = 0.23; P = 0.92$) and to bendiocarb (ANOVA: df = 1; $F = 0.18; P = 0.71$). The difference in percentage mortality between pyrethroid and carbamate assays and sites was also not statistically significant (ANOVA: df = 1; $F = 4.39; P = 0.13$).

Table 16: Twenty four hour mortality and resistance status of 3-5 day old adult female F1 *Anopheles funestus* progeny from Burma Valley and Zindi recorded during WHO susceptibility bioassay carried out in February 2014

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>N‡</th>
<th>No. of tubes/replicates</th>
<th>% mortality mean ± SE</th>
<th>Resistance status</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Burma Valley</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05% Lambda-cyhalothrin</td>
<td>100</td>
<td>4</td>
<td>9.0 ± 1.8</td>
<td>R</td>
<td>4-13.8</td>
</tr>
<tr>
<td>0.05% Deltamethrin</td>
<td>87</td>
<td>4</td>
<td>12.6 ± 0.8</td>
<td>R</td>
<td>10.8-14.7</td>
</tr>
<tr>
<td>0.5% Etofenprox</td>
<td>90</td>
<td>4</td>
<td>3.3 ± 0.7</td>
<td>R</td>
<td>1.6-4.9</td>
</tr>
<tr>
<td>0.1% Bendio carb</td>
<td>98</td>
<td>4</td>
<td>25.5 ± 1.4</td>
<td>R</td>
<td>21.3-28.8</td>
</tr>
<tr>
<td>4% DDT</td>
<td>100</td>
<td>4</td>
<td>100</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>1.0% Pirimiphos methyl</td>
<td>100</td>
<td>4</td>
<td>100</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Untreated control</td>
<td>129</td>
<td>5</td>
<td>0.8 ± 0.4</td>
<td>S</td>
<td>0-1.8</td>
</tr>
<tr>
<td><strong>Zindi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05% Lambda-cyhalothrin</td>
<td>107</td>
<td>5</td>
<td>4.7 ± 0.3</td>
<td>R</td>
<td>3.8-5.7</td>
</tr>
<tr>
<td>0.05% Deltamethrin</td>
<td>92</td>
<td>4</td>
<td>16.3 ± 0.8</td>
<td>R</td>
<td>14.0-18.4</td>
</tr>
<tr>
<td>0.5% Etofenprox</td>
<td>83</td>
<td>4</td>
<td>13.3 ± 0.6</td>
<td>R</td>
<td>11.8-14.9</td>
</tr>
<tr>
<td>0.1% Bendio carb</td>
<td>100</td>
<td>4</td>
<td>8.0 ± 1.0</td>
<td>R</td>
<td>5.5-10.2</td>
</tr>
<tr>
<td>4% DDT</td>
<td>114</td>
<td>5</td>
<td>100</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>1.0% Pirimiphos methyl</td>
<td>96</td>
<td>4</td>
<td>100</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Untreated control</td>
<td>122</td>
<td>5</td>
<td>0.0</td>
<td>S</td>
<td></td>
</tr>
</tbody>
</table>

‡, Total number of insects exposed; R, resistant; S, susceptible

7.3.4 Knockdown effect of insecticide on F1 *Anopheles funestus* progeny females

Common similarities were observed in KD$_{50}$ and KD$_{95}$ values between the pyrethroids (lambda-cyhalothrin and deltamethrin) and carbamates (bendiocarb), and between organochlorines (DDT) and organophosphates (pirimiphos-methyl) in both Burma Valley and Zindi (Table 17). The knockdown effects of the four classes of insecticides tested over one hour showed more rapid
knockdown rate for DDT and pirimiphos-methyl than the other two classes of insecticides (Table 17). DDT knocked down 50 and 95% of the mosquitoes from both sites within 50 and 60 min of exposure, respectively. Fifty per cent and 95% knockdown was achieved within 50 and 80 min, respectively, for mosquitoes collected from Zindi when exposed to pirimiphos-methyl. There was loss of knockdown effect on all samples from both sites when exposed for 80 min to lambda-cyhalothrin and deltamethrin and bendiocarb.

Table 17: Association between percentage 24-hour mortality and knockdown (KD) time using WHO test tubes

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Site</th>
<th>Mortality (%)</th>
<th>KD$_{50}$ (min)</th>
<th>KD$_{95}$ (min)</th>
<th>Resistance status</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05% Lambda-cyhalothrin</td>
<td>Burma Valley</td>
<td>9.0</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>4.7</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td>0.05% Deltamethrin</td>
<td>Burma Valley</td>
<td>12.6</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>16.3</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td>0.1% Bendiocarb</td>
<td>Burma Valley</td>
<td>25.5</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>8.0</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td>0.5% Etofenprox</td>
<td>Burma Valley</td>
<td>3.3</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>13.3</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td>4% DDT</td>
<td>Burma Valley</td>
<td>100</td>
<td>50</td>
<td>60</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>100</td>
<td>40</td>
<td>50</td>
<td>S</td>
</tr>
<tr>
<td>1% Pirimiphos-methyl</td>
<td>Burma Valley</td>
<td>100</td>
<td>30</td>
<td>60</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>100</td>
<td>50</td>
<td>80</td>
<td>S</td>
</tr>
</tbody>
</table>

S, susceptible; R, resistant; KD, knockdown; KD$_{50}$, knockdown rate for 50% of mosquitoes; KD$_{95}$, knockdown rate for 95% of mosquitoes; No KD, loss of knockdown effect (<20% of mosquitoes knocked down after 1-hour exposure).

7.3.5 Insecticide resistance intensity in *Anopheles funestus* F1 female progeny

*Anopheles funestus* exhibited various levels of knockdown effects after eight-hour exposure to insecticides, with highest sensitivity observed in bendiocarb for the two localities (Table 18). In
both areas, there were no statistically significant differences in responses among lambda-cyhalothrin, deltamethrin and etofenprox over the entire eight-hour observation period (ANOVA: df = 5; $F = 2.39; P = 0.11$). Although knockdown rate for deltamethrin in Burma Valley and Zindi were observed from 30 and 80 min, respectively, the sensitivity of the mosquitoes to the insecticide could not stretch beyond 90% knockdown effect within an eight-hour monitoring period (Figures 3 and 4). Similarly, observations on lambda-cyhalothrin and etofenprox showed percentage knockdown rate of less than 100% for the entire experimental period in both sites.

Table 18: Percentage knockdown after an 8-hour exposure and KD$_{50}$ values of F1 adult progeny raised from female Anopheles funestus collected in Burma Valley and Zindi

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Location</th>
<th>N§</th>
<th>% Knockdown (mean ± SE)</th>
<th>KD$_{50}$ (min)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05% Lambda-cyhalothrin</td>
<td>Burma Valley</td>
<td>58 (3)</td>
<td>84.4 ± 0.4</td>
<td>240</td>
<td>83.7-85.4</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>110 (5)</td>
<td>92.7 ± 0.8</td>
<td>300</td>
<td>90.2-94.2</td>
</tr>
<tr>
<td>0.05% Deltamethrin</td>
<td>Burma Valley</td>
<td>100 (4)</td>
<td>90.0 ± 1.4</td>
<td>300</td>
<td>86.2-93.7</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>75 (3)</td>
<td>84.0 ± 1.4</td>
<td>240</td>
<td>80.9-86.6</td>
</tr>
<tr>
<td>0.1% Bendiocarb</td>
<td>Burma Valley</td>
<td>39 (2)</td>
<td>100</td>
<td>120</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>105 (5)</td>
<td>100</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>0.5% Etofenprox</td>
<td>Burma Valley</td>
<td>24 (1)</td>
<td>66.7 ± 0.6</td>
<td>300</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>41 (2)</td>
<td>70.7 ± 0.6</td>
<td>480</td>
<td>69.8-71.6</td>
</tr>
</tbody>
</table>

§, number of tubes/replicates in parentheses
Figure 3: Percentage knockdown times of *Anopheles funestus* from Burma Valley following 8-hour exposures to various insecticides

Figure 4: Percentage knockdown times of *Anopheles funestus* from Zindi following 8-hour exposures to various insecticides

7.4 Discussion

The status of susceptibility/resistance to lambda-cyhalothrin, deltamethrin, etofenprox, bendiocarb, DDT and pirimiphos-methyl was evaluated in *An. funestus* wild populations and F1 progeny females collected from Burma Valley and Zindi in Zimbabwe. With guidance from
WHO (2013a) protocol for characterizing insecticide resistance, where susceptibility is defined by mortality rates above 98% and resistance by mortality less than 90% 24-hours post exposure, this study provides evidence that *An. funestus* populations from both sites were resistant to pyrethroids and carbamates, but susceptible to DDT and pirimiphos-methyl. The information is important in malaria vector control operations, which in Zimbabwe are strongly dependent on the use of insecticides in IRS and LLINs.

Zimbabwe has been using DDT for both tsetse fly and malaria vector control since 1949 (Munhenga *et al.*, 2008). Currently, DDT is being applied for malaria vector control in the low veld zones of Zimbabwe (< 600 m altitude), and pyrethroids, especially lambda-cyhalothrin and deltamethrin, are used interchangeably to cover the middle veld zones (600-1,200 m altitude). Continuous application of DDT and alternating lambda-cyhalothrin with deltamethrin might increase selection pressure, resulting in early loss of sensitivity in vector populations. Choi *et al.* (2014) reported *An. funestus* resistance to lambda-cyhalothrin, deltamethrin and bendiocarb for the first time in the area adjacent to the Zindi collecting site, but no DDT and organophosphate-resistant populations were detected in that area. These findings are consistent with the results of the present work, which showed resistance in *An. funestus* to pyrethroids and bendiocarb. In both areas, there were no statistically significant differences in 24-hour mortality in mosquitoes exposed to lambda-cyhalothrin, deltamethrin, etofenprox, and bendiocarb. The detection of deltamethrin and lambda-cyhalothrin resistance in the *An. funestus* populations in Burma Valley and Zindi is a worrying result as these are the most common insecticides applied interchangeably by NMCP in Zimbabwe to prevent malaria transmission in the study areas.

The build-up of pyrethroid and carbamate resistance in the *An. funestus* populations from the two study areas is not clear. Most probably the increase in the selection pressure exerted by pyrethroids may be attributed to their continuous use in public health, agriculture and at household level to control domestic pests. Bendiocarb resistance may be mainly associated with application in agriculture, which is a major source of livelihood in both study areas. The incrimination of agricultural use of pesticides in the selection pressure against *Anopheles* populations has also been reported in several countries in West Africa (Akogbeto *et al.*, 2006; Namountougou *et al.*, 2012). Since pyrethroid resistance has been reported to result mainly from
agricultural application, it is likely that such resistance will develop regardless of the organized use of pyrethroids in properly managed malaria control programmes (Chandre et al., 1999).

Results of the present work agree with other studies that reported pyrethroid and bendiocarb resistance in the An. funestus populations from Mozambique (Casimiro et al., 2006), Ghana (Okoye et al., 2008), Malawi (Hunt et al., 2010), and Zambia and Zimbabwe (Choi et al., 2014). The reported occurrence of permethrin and DDT resistance in malaria vectors in Gokwe District (Munhenga et al., 2008) was not detected in An. gambiae s.l. populations from 16 sentinel sites (Burma Valley and Zindi included) following a nationwide study (Lukwa et al., 2014). Resistance to pyrethroids generally confers cross-resistance to other insecticides with the same mode of action, thus limiting the alternative choices of effective insecticide (Jirakanjanakit et al., 2007). The lack of cross-resistance between pyrethroids and DDT observed in this study is consistent with the work of Coetzee and Koekemoer (2013), which reported that pyrethroid resistance in An. funestus, is mostly conferred fully or partially by monooxygenases (P450) in most countries in southern Africa. Further, it appears there is no knockdown resistance (kdr) gene in Southern African An. funestus to date (Coetzee and Koekemoer, 2013), as is also clearly indicated by the observation of this work. However, cross-resistance to pyrethroids and DDT has been reported in most mosquito species of public health importance collected from other countries as a result of a kdr gene (Hemingway and Ranson, 2000; Brengues et al., 2003).

In addition to mortality, knockdown time might be a valuable tool for the early detection of reduced susceptibility, although there are no WHO standards on knockdown time specified to indicate resistance. Knockdown time has long been accepted as an indicator of susceptibility in vector mosquitoes to insecticides. The time provides initial data on the possible involvement of kdr gene (Chandre et al., 1999), although high frequency of a resistant gene does not necessarily translate into resistance in Anopheles populations (Djegbe et al., 2011).

The results of this work have demonstrated elevated knockdown time for all insecticides tested, with the increase more pronounced in pyrethroids and carbamates than DDT and organophosphate. However, there was no difference between the time required to knockdown 100% of the mosquitoes due to DDT and pirimiphos-methyl from the two sites. Although this study detected no resistance to DDT and pirimiphos-methyl, the KD50 and KD95 values

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obtained from both sites for these two insecticides appear to be abnormally high, ranging from 30-80 min to knockdown 100% of the specimens. These results may be an indication of future problems with the application of DDT and pirimiphos-methyl in Burma Valley and Zindi.

The high survival rates and the increased knockdown time detected in this study raise the question of whether mosquitoes are withstanding higher concentrations of insecticide or whether longer exposure times are needed. To address the latter question, a resistance intensity test was included in the current study in order to determine the strength of resistance, although it is not the standard method of measuring resistance. Currently, the standard methods of anopheline bioassays are the WHO (2013a) tube assay and the CDC (2010) bottle assay. Although the two methods generally agree on resistance frequencies, there has been no agreement on the application of resistance intensity test as a standard tool for measuring insecticide resistance in mosquito populations.

The CDC (2010) bottle bioassay method recommends the extension of diagnostic time to two hours in order to evaluate intensity of resistance, but does not give criteria for assessing resistance intensity. Within two hours of continuous exposure, An. funestus from both sites showed mortality of less than 40% to lambda-cyhalothrin, deltamethrin and etofenprox, with about 80% to bendiocarb, suggesting a high level of resistance to the pyrethroids. A problem with not achieving 100% knockdown after an eight-hour exposure time to all insecticides used, save for bendiocarb, might indicate serious resistance intensity. At operational level, this poses a major challenge as it is not clear whether a mosquito rests continuously for eight hours on a sprayed surface or on a treated net, taking into cognisance the repellency and irritancy properties contained in various insecticides.

Focusing on the pattern emerging from the two study sites, it is clear that An. funestus resistance to pyrethroids and carbamates, and susceptibility to DDT and pirimiphos-methyl, are firmly indicated. The resistance in the An. funestus populations detected in this study has serious implications for the current insecticide-based malaria control efforts being undertaken in the study areas. The results seem to suggest the need for urgent and effective insecticide resistance management strategies necessary for the prevention of rapid build-up of resistance across all four commonly used classes of insecticides to control vectors of public health importance.
CHAPTER 8

REVIEW

A REVIEW OF NEW CHALLENGES AND PROSPECTS FOR MALARIA ELIMINATION IN MUTARE AND MUTASA DISTRICTS

8.1 Introduction

Following the aborted Global Malaria Eradication campaign in the 1960s-1970s, malaria received little international attention over the subsequent years until recently (RBM, 2008). After the launch of the RBM programme in 1998, most countries with endemic malaria, especially in Africa, made substantial progress in their malaria control interventions. Currently, it appears commitment has greatly improved, and partnerships exist to accelerate and sustain malaria control and elimination to achieve national, regional and global malaria targets and the malaria-related Millennium Development Goals (MGDs) (WHO, 2010a). Malaria elimination has been defined as permanent reduction to zero incidences of locally contracted cases (WHO, 2008). The malaria target under MGD 6 (halting and beginning to reverse the incidence of malaria by 2015) has been met and 55 countries are on track to reduce their malaria burden by 75% in line with the World Health Assembly’s target of 2015. Malaria mortality decreased by 47% between 2000 and 2013 globally, and by 54% in the WHO African region, with an increasing number of countries striving towards malaria elimination (WHO, 2015a). This progress is primarily attributed to scaled-up vector control interventions, especially indoor residual spraying (IRS) and long lasting insecticidal nets (LLINs), as well as improved malaria diagnosis and effective treatment.

In Zimbabwe, vector control is a central, critical component of all malaria control strategies and the use of IRS and LLINs has increased immensely over the past decade as part of an effort towards universal coverage of all populations at risk of contracting the disease. If a universal coverage and greater than 80% use of IRS or LLINs by populations at risk of malaria are attained, consolidated and maintained, malaria transmission will be significantly reduced (WHO, 2010a). Over the years, new challenges have emerged, further complicating the goal of eliminating malaria. Despite Zimbabwe being a member of Elimination 8 (E8) countries, the emerging threats and prospects for a successful shift from malaria control to elimination in Mutare and Mutasa Districts are not well understood. The article reviews work on malaria parasites, vector species composition, insecticide resistance and responses in vector mosquitoes following prolonged use of IRS and LLINs. In this review, the aim was to identify and describe common emerging challenges and prospects for malaria elimination in Mutare and Mutasa Districts, Zimbabwe, where substantial and sustained strides have been made towards control.
8.2 Selected districts and data collection

Mutare and Mutasa Districts of Manicaland Province, Zimbabwe, are among some of the few areas for which historical entomological data and related information is readily available. The intensity of malaria transmission in the two districts differs considerably, with Mutasa District always in the lead. In the two districts, 95% of all malaria cases are caused by *Plasmodium falciparum* (Lukwa *et al.*, 2014) and primarily transmitted by *Anopheles funestus* (Sande *et al.*, 2015a). The disease is seasonal, but prone to sporadic epidemics, and is considered a public health problem in the two districts. Indoor residual spraying and LLINs are the major vector control strategies employed to combat malaria. In 2014, IRS protected over 80% of people at risk of malaria in the two districts (Mberikunashe, unpublished data). However, the population protected by use of mosquito nets is not clear.

Information on mosquito vector control, behaviour, and epidemiology in Zimbabwe is available from work by various researchers as well as unpublished data from sources such as the National Malaria Control Programme (NMCP), National Institute of Health Research (NIHR), national archives and academic institutions. Work by Mpofu (1985), Taylor and Mutambu (1986), Masendu *et al.* (2005) and Sande *et al.* (2015a), reported extensively on malaria species composition and relative abundance in various regions of Zimbabwe. Leeson (1931), Alves and Blair (1955), Mabaso *et al.* (2004) and Munhenga (2010) documented the history of vector control through use of IRS as far back as the 1940s. Masendu (1996), Dandalo (2007) and Sande *et al.* (2016a, 2016b) reported on the resting and biting behaviour of vector mosquitoes from 1996 to 2014 in various parts of Zimbabwe. Some changes in vector behaviour including resting and biting have been attributed to prolonged use of IRS and LLINs as was reported for the major vector *An. gambiae s.s.* in Bioko Island, Equatorial Guinea (Reddy *et al.*, 2011).

8.3 Malaria situation prior to the house spraying and mosquito net era

Prior to the implementation of IRS and/or LLINs, endemicity of malaria was shown to be markedly influenced by altitude, varying from hyperendemic in the low altitude areas (elevation less than 700 m) to hypoendemic or completely absent on the central watershed (elevation more than 1,200 m) (Leeson, 1931; Alves and Blair; 1955; Masendu *et al.*, 2005). Malaria
transmission was intense, yet clearly seasonal, peaking from February to April, and the geographical distribution was more extensive, with sporadic epidemics in some areas (Leeson, 1931; Alves and Blair, 1955). Random malariometric surveys, especially spleen and parasite rates were carried out as pre-control strategies in selected districts (Alves and Blair, 1955).

8.4 Malaria situation after the introduction of house-spraying and mosquito nets

Over the last decade, Zimbabwe has recorded a steady annual decline in malaria morbidity, from an annual incidence of 55 cases per 1,000 populations in 2003, to 29 cases per 1,000 populations by the end of 2013. Malaria deaths decreased from approximately 3,000 in the early 2000s to about 300 people per annum in recent years (GF, 2014). A clear reduction in malaria burden was observed in the southern and central parts of Zimbabwe, with Matabeleland South Province recording malaria cases of less than 1 per 1,000 populations in 2012 (GF, 2014). From 2013, the NMCP upgraded Matabeleland South Province from implementing malaria control activities to pre-elimination. All gains have coincided with widespread adoption of various malaria control strategies, especially IRS, LLINs, and early diagnosis and effective treatment. It appears the challenge is that most of the milestones achieved in malaria control over the years are unevenly distributed and breakable in Zimbabwe, especially in Mutare and Mutasa Districts. However, from 2003 to 2013, the malaria incidence, though declining, remained relatively high in Mutare (19.5%, range 4.9-62.3%) and in Mutasa (50.9%, range 11.2-88.1%) (DHIS 2, unpublished data).

8.5 Status of malaria elimination

In 2009, a meeting was held by Ministers of Health of eight Southern African countries, the Malaria Elimination 8 (E8), in Windhoek, Namibia, to deliberate on the mechanisms and partnerships necessary for malaria elimination in their sub-region (SARN, 2010). A subsequent E8 inaugural meeting was held in Maputo, Mozambique in 2010, which served as a forum for the Ministers of Health of the eight countries to coordinate efforts and assess progress made towards malaria elimination (SARN, 2010). Four frontline countries (Botswana, Namibia, South Africa and Swaziland) for E8 were positioned to immediately move from malaria control to elimination, while the remaining four (Angola, Mozambique, Zambia and Zimbabwe) were expected to
consolidate malaria control, supporting the frontline countries and preparing the transition to malaria elimination phase.

Resulting from the Maputo meeting, the malaria situation was assessed in all eight rural provinces of Zimbabwe following WHO guidelines (WHO, 2007a). The criterion for zonal classification into malaria programme phases and milestones on the path to malaria elimination was followed, with control and consolidation (slide positive rate < 5% in all fever cases), pre-elimination (< 1 case/1000 population at risk per year), elimination (0 local acquired cases), and prevention of reintroduction (WHO certification, 3 years without local transmission) (WHO, 2007a). The first province in Zimbabwe to implement activities under malaria pre-elimination/elimination phase was Matabeleland South in 2013. Currently, Matabeleland North, Midlands and Mashonaland West Provinces have also been promoted to implement malaria pre-elimination/elimination activities in some districts with effect from 2015. The remaining four rural provinces (Masvingo, Mashonaland Central, Mashonaland East and Manicaland) are strongly expected to continue to implement activities in the control phase, but under tight surveillance for a possible move to elimination.

8.6 Parasite and vector species composition

The predominant malaria parasite species in Zimbabwe is *P. falciparum* which accounts for over 95% of malaria cases in the country (Lukwa *et al*., 2014). The other few malaria cases are caused by *P. malariae* and *P. ovale*. The most important vector species which transmit human malaria in Africa belong to members of the *An. gambiae* complex and the *An. funestus* group. In Zimbabwe, Mpofu (1985), Taylor and Mutambu (1986), and Masendu *et al*. (2005), confirmed the presence of four members of the *An. gambiae* complex: *An. gambiae*, *An. arabiensis*, *An. merus* and *An. quadriannulatus*. More recently, Sande *et al*. (2015a) in Mutare and Mutasa Districts, Zimbabwe, reported the sympatric occurrence of *An. arabiensis* and *An. quadriannulatus*. Within the *An. gambiae* complex, *An. arabiensis* and *An. gambiae* are the major human malaria vectors in sub-Saharan Africa (Lanzaro and Lee, 2013).

Previous studies on the *An. funestus group* by Evans and Leeson (1935) in Zimbabwe, reported the presence of *An. funestus*, *An. leesoni* and *An. confusus*. Green and Hunt (1980) reported *An.*
funestus, An. parensis and An. aruni in sympatry in various parts of Zimbabwe. More recently, An. funestus and An. leesoni sibling species were detected in Mutare and Mutasa Districts (Choi et al., 2014; Sande et al., 2015a). In the An. funestus group, An. funestus is the only member that is implicated as an important vector of malaria in sub-Saharan Africa (Coetzee et al., 2000).

From as far back as the early 1970s, An. arabiensis was noted to be the primary vector of malaria in Zimbabwe while An. gambiae and An. funestus are secondary vectors (Taylor and Mutambu, 1986; Masendu et al., 2005). A nationwide vector distribution survey in Zimbabwe in 2005 reported the presence of An. funestus only at Buffalo Ranch in Chiredzi District of Masvingo Province, in the southern region of the country (Masendu et al., 2005). The scarcity of An. funestus was attributed to its elimination following decades of IRS. Interestingly, a 2013-2014 study on vector species composition in Mutare and Mutasa Districts showed the resurgence of An. funestus in the two districts (Choi et al., 2014; Sande et al., 2015a). The two studies demonstrated the shift in dominance of An. funestus from a secondary to a primary vector (95.4%), with An. arabiensis being relegated to a secondary vector (4.6%) in the two districts. In the absence of recent species composition data from other parts of Zimbabwe, the resurgence of An. funestus in Mutare and Mutasa could be more widespread than previously thought.

The supremacy of An. funestus in Mutare and Mutasa Districts is an emerging challenge to malaria control and elimination, primarily because it is a more efficient vector than An. arabiensis (Gillies and De Meillon, 1968; Bruce-Chwatt, 1985). Additionally, An. funestus is fairly difficult to collect in its larval stage and its adaptability to field insectary and laboratory conditions is poor, leading to inconsistent entomological studies using this species. Regular entomological monitoring of vector species is of paramount importance to malaria control and elimination in any setting. The predominantly indoor resting and host-seeking traits of An. funestus reported by Pates and Curtis (2005) in various parts of Africa, Sande et al. (2016a, 2016b) in Mutare and Mutasa set opportunities for its control using IRS or LLINs, with prospects of achieving the malaria elimination goal when combined with other malaria interventions.
8.7 House spraying and use of mosquito nets for malaria control

In Zimbabwe, IRS was started as a pilot study as far back as the 1940s using DDT and then BHC (Alves and Blair, 1953; Alves and Blair, 1955; Mabaso et al., 2004), and is currently the mainstay of malaria vector control in the country. In 1986, following years of DDT use, deltamethrin was evaluated in Zimbabwe in experimental huts and the residual effect was found acceptable for malaria vector control (Taylor and Mutambu, 1986). Again in 1986, micro-encapsulated deltamethrin was tried under field conditions and recommended for widespread spraying in the country (Taylor and Mutambu, 1986). Later, in 1990, lambda-cyhalothrin was tested in a small community and the residual activity was found to be comparable to deltamethrin and suitable for nationwide use.

Since the 1940s, residual spraying with DDT and more recently with pyrethroids has been the NMCP’s major vector control intervention with the aim of reducing malaria burden (Sande et al. 2015a). Over the past five years, implementation of IRS followed WHO’s recommendation of spray and population coverage of at least 80%. The spray coverage and the proportion of population protected from 2009 to 2014 are shown on Table 19, with above 80% spray and population protected coverage overall. This milestone, if maintained, might be an opportunity for malaria elimination in the near future for the two districts, especially adhering to the recommendations by the WHO (2007a) to target all villages with annual parasite index (API) of more than 5 cases per 1,000 populations per annum. However, the major challenge is that the selection criteria of villages to be sprayed in each district by the Zimbabwe’s NMCP are not based on API, but are resource-based, leaving some villages with API of > 5% in Mutare and Mutasa Districts unsprayed. Hence, sporadic malaria outbreaks experienced in Mutare and Mutasa Districts in recent years have occurred in unsprayed villages with API of > 5% (Mberikunash, personal communication), posing a serious operational challenge to malaria control and elimination. Moreso, part of the challenge is with the sprayers themselves, where, in most instances, the standard compression sprayers and mode of application depend entirely on the ability and diligence of the spray operator to deliver the correct dose in the right location (Knapp et al., 2015). While the IRS programme in Mutare and Mutasa uses WHO’s recommended compression sprayers, NMCP has not been able to consistently provide constant
flow valve (CFV) for each sprayer over the years. The CFVs maintain a uniform application rate as the pressure in the tank falls and enhances overall efficiency of spraying (Kumar et al., 2013).

Table 19: House spraying coverage and population protected in Mutare and Mutasa Districts from 2009 to 2014

<table>
<thead>
<tr>
<th>Year</th>
<th>Mutare</th>
<th>Mutasa</th>
<th>Manicaland</th>
<th>Zimbabwe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% cov.</td>
<td>% pop. prot.</td>
<td>% cov.</td>
<td>% pop. prot.</td>
</tr>
<tr>
<td>2009</td>
<td>98</td>
<td>99</td>
<td>99</td>
<td>86</td>
</tr>
<tr>
<td>2010</td>
<td>95</td>
<td>97</td>
<td>92</td>
<td>95</td>
</tr>
<tr>
<td>2011</td>
<td>89</td>
<td>100</td>
<td>86</td>
<td>93</td>
</tr>
<tr>
<td>2012</td>
<td>84</td>
<td>93</td>
<td>84</td>
<td>80</td>
</tr>
<tr>
<td>2013</td>
<td>80</td>
<td>95</td>
<td>87</td>
<td>85</td>
</tr>
<tr>
<td>2014</td>
<td>96</td>
<td>84</td>
<td>86</td>
<td>92</td>
</tr>
</tbody>
</table>

% cov. = percentage coverage; % pop. prot. = percentage population protected

To achieve the desired results in malaria control using IRS, Zimbabwe has been employing the WHO’s recommended insecticides. DDT and BHC were used from 1945 to 1962, BHC independently in 1972 to 1973, DDT independently from 1974 to 1987, deltamethrin and lambda-cyhalothrin from 1988 to 2000 (Mabaso et al., 2004). Insecticides used from 2001 to 2013 are shown on Table 20 and it is clear that the NMCP’s IRS used pyrethroids for 13 years consecutively in Mutare and Mutasa Districts. The IRS spray programme switched to organophosphates in 2014 following results of insecticide resistance studies in An. funestus in Mutare and Mutasa Districts (Choi et al., 2014, Sande et al., 2015b). It is clear from Table 8.2 that insecticide classes were not rotated to effectively manage insecticide resistance. The lack of insecticide rotation suggests unavailability or non-use of insecticide resistance management plan which is a major challenge in achieving the malaria elimination goal.

Traditionally, mosquito nets played a much lesser role than IRS until the initiation of LLIN campaigns under the universal coverage goal over the past few years. To fully implement the two vector control interventions, Zimbabwe had no clear guidance to inform provinces to balance the deployment strategies of LLINs and IRS following recommendations by the WHO (2014), especially the effectiveness of combining versus either IRS or LLINs alone, as well as the
problem of introducing the second intervention as a means of compensating for the deficiencies in the implementation of the first strategy.

Table 20: Insecticides, formulations and amounts used in Mutare and Mutasa Districts for IRS from 2001 to 2014

<table>
<thead>
<tr>
<th>Year</th>
<th>Insecticide</th>
<th>Class</th>
<th>Formulation</th>
<th>Amount used</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroids</td>
<td>10 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2002</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroids</td>
<td>10 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2003</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroids</td>
<td>10 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2004</td>
<td>Deltamethrin</td>
<td>Pyrethroids</td>
<td>5 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2005</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>10 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2006</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>10 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2007</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>10 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2008</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>10 WP</td>
<td>26,412 sachets</td>
</tr>
<tr>
<td>2009</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>10 WP</td>
<td>27,564 sachets</td>
</tr>
<tr>
<td>2010</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>5 WP</td>
<td>36,348 sachets</td>
</tr>
<tr>
<td>2011</td>
<td>Deltamethrin</td>
<td>Pyrethroid</td>
<td>5 WP</td>
<td>42,706 sachets</td>
</tr>
<tr>
<td>2012</td>
<td>Deltamethrin</td>
<td>Pyrethroid</td>
<td>5 WP</td>
<td>39,464 sachets</td>
</tr>
<tr>
<td>2013</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>10 WP</td>
<td>36,643 sachets</td>
</tr>
<tr>
<td>2014</td>
<td>Pirimiphos-methyl</td>
<td>Organophosphate</td>
<td>300 CS</td>
<td>37,927 bottles</td>
</tr>
</tbody>
</table>

Despite reports which showed more than 90% distribution coverage of LLINs in Mutare and Mutasa Districts (Mberikunashe, unpublished data); net utilization data could not be easily accessed. However, utilisation of mosquito nets amongst the population at risk in Manicaland Province was 47.5% in 2012 (Zimbabwe Malaria Indicator Survey, unpublished data), 33.5% short of the WHO’s 80% coverage for impact. The low utilisation of nets in the province may suggest equally low rate of utilisation of the product in Mutare and Mutasa Districts. This poses a serious challenge as the effectiveness of mosquito nets to combat malaria largely depends on their utilisation by majority of people at risk.

### 8.8 Resistance to antimalarial medicines and insecticides

The real need to intensify IRS arose when the first case of chloroquine resistance was confirmed from the Zambezi Valley, Zimbabwe in 1984 (Dallas et al., 1984). Chloroquine was then the first line antimalarial medicine to treat uncomplicated malaria in Zimbabwe (Mharakurwa and Mugochi, 1994). By 1989, chloroquine-resistant infections had been demonstrated in most parts
of the endemic zones of the country, with varying types and levels of resistance (Makanda, 1987; Simooya et al., 1992; Mharakurwa and Mugochi, 1994), and alarming resistance levels being reported in a localised area of Gokwe District.

Following confirmation of chloroquine resistance in several parts of Zimbabwe (Makanda, 1987; Simooya et al., 1992; Mharakurwa and Mugochi, 1994), chloroquine was replaced by a free combination of chloroquine and sulfadoxine-pyrimethamine (SP) as first line of antimalarial medicine in the early 2000s. Subsequent studies indicated rising failure of chloroquine and SP combination and these were replaced by artemesinin-based combination therapy (ACT) in 2004. The ACT antimalarials were rolled out fully in 2007/8 (Zimbabwe Malaria Programme Review, unpublished data). These introductions and replacement of antimalarial medicines highlight the serious challenges to malaria control and elimination in Zimbabwe. This situation will be exacerbated when the same area experiences insecticide resistance in major malaria vectors.

Only four classes of insecticides are approved by WHO to control malaria vector mosquitoes using house spraying (WHO, 2012). These are pyrethroids, organochlorines, organophosphates and carbamates. At present, all WHO-recommended LLINs (WHO, 2012) are treated with pyrethroids. The high dependence on pyrethroid-based malaria control has increased the selection pressure for insecticide resistance in malaria vectors. Even though insecticides have been used for a very long period in Zimbabwe, there are very few instances where resistance has been recorded (Munhenga et al., 2008). Early reports of insecticide resistance in An. arabiensis appeared in the 1980s in Chiredzi District and showed BHC resistance (Green, 1982). Masendu et al. (2005) and Munhenga et al. (2008) reported resistance in An. arabiensis to DDT and permethrin from Gokwe District. No further insecticide resistance was documented in Zimbabwe till recently when pyrethroid and carbamate resistance was reported in An. funestus in Mutare and Mutasa District Choi et al., 2014; Sande et al., 2015b). Interestingly, the same studies showed that An. funestus populations were susceptible to both DDT (organochlorine) and pirimiphos-methyl (organophosphates).

While the emergence of insecticide resistance in An. funestus in Mutare and Mutasa Districts is likely to reverse the gains made in malaria control, the lack of cross-resistance observed between pyrethroids and DDT is an opportunity for malaria control and elimination, as DDT could be an
option to replace pyrethroids for house-spraying. Following evidence of pyrethroid and carbamate resistance in *An. funestus* collected from Mutare and Mutasa Districts, Zimbabwe’s NMCP changed insecticide used for IRS from pyrethroids to pirimiphos-methyl 300 CS (organophosphate) in 2014. The major challenge with the use of pirimiphos-methyl 300 CS is that the cost is comparatively high and might be unsustainable to government and malaria stakeholders, leading to possible reversal of milestones gained in malaria control.

### 8.9 Resting and biting behaviour of vectors *visa-vis* house-spraying and mosquito nets

The effectiveness of IRS and LLINs to prevent malaria transmission largely depends on resting and biting behaviours of the vectors. Indoor house spraying is effective against indoor resting mosquitoes, whereas LLINs control malaria vectors that bite indoors. Although several studies have shown the efficacy of IRS and LLINs in reducing malaria incidence in almost all settings (WHO, 2014b; Pluess *et al.*, 2010; Lengeler, 2004), outdoor transmission poses a serious challenge to malaria control and elimination.

From 1996 to 2015, four studies reported on resting and biting behaviour of mosquitoes collected from Gokwe and Binga Districts (Masendu, 1996), Gokwe South District (Dandalo, 2007), and Mutare and Mutasa Districts (Sande *et al.*, 2016a, 2016b) in Zimbabwe. The first study (Masendu, 1996) showed that the principal vector *An. arabiensis* was partially exophilic, consequently, it might not be fully amenable to control by indoor application of residual insecticides, posing a challenge to malaria control. The second (Dandalo, 2007) demonstrated that the resting behaviour of *An. gambiae* complex was mainly exophilic, while its peak indoor biting activity occurred at 22:00 hours, probably some people would still be out of bed and not protected from mosquito bite by nets. Mosquito outdoor biting behaviour was not evaluated in this study.

The third study (Sande *et al.*, 2016a) conducted in Mutare and Mutasa Districts established that 84% of the *An. funestus* populations were endophilic, with a lower percentage exhibiting exophilic traits (16%). Of those collected indoors, 90% were collected on sprayable habitats (walls and roofs/ceiling) and 10% on unsprayable surfaces (furniture and other household
goods). Of those collected on sprayable surfaces, 56% were collected on the roofs, with 44% on the walls. For the past five years, the NMCP could not consistently spray roofs/ceiling owing to non-availability of extension lances to spray surfaces higher than 3.5 m from the ground level. Failure to spray roofs/ceiling on which the majority of mosquito species rest is a cause for concern to malaria control and elimination programmes in Mutare and Mutasa Districts.

The fourth study (Sande et al., 2016b) reported trapping An. funestus populations and An. gambiae s.l. more abundantly indoors (68%) than outdoors (32%), suggesting that malaria could be interrupted through the use of LLINs by the majority of residents in the study sites. However, the observed variable nocturnal flight activity rhythms of An. funestus in Mutare and Mutasa Districts, with two peaks during the night; between 22:00-23:00 and 02:00-04:00 hours, is a cause for concern. Both peaks suggest that malaria transmission might be maintained despite net ownership and use as this was a period when probably a fairly small proportion of the rural population might not have gone to bed yet or might have got out of bed already for early morning household chores.

8.10 Conclusion

Critical emerging challenges to malaria control and elimination exist in Mutare and Mutasa Districts of Manicaland Province in Zimbabwe. The emergence of resistance to antimalarial medicines and insecticides, failure to spray all villages with an API of > 5%, unavailability of clear guidelines on the deployment of IRS and LLINs, the use of alternatives and possible more costly insecticide in IRS to maintain the required level of vector control interventions, as well as the resurgence of one of the most efficient malaria vector, An. funestus, non-spraying of roofs/ceiling where majority of mosquitoes prefer to rest, and possible outdoor transmission, continue to threaten the milestones gained towards malaria elimination. However, realising the emerging challenges to achieve malaria elimination goal does not provide justification for opposition to progressing towards regional goal and agenda of malaria elimination. The predominant endophilic behaviour and high indoor CDC catches of An. funestus, lack of cross resistance between pyrethroids and DDT in An. funestus, as well as scaled-up malaria control interventions, especially high house-spray coverage or LLINs distribution, and the Zimbabwe
NMCP’s commitment to E8 agenda create prospects for malaria elimination in Mutare and Mutasa Districts in the near future.

Evidence presented in this review suggests that selection of malaria intervention strategies in Mutare and Mutasa Districts, especially antimalarial medicines, insecticides for IRS and use of pyrethroid-based LLINs should be based on susceptibility status to antimalarials and insecticides, as well as resting and biting behaviour of the vector mosquitoes. These aspects are important to achieve global health agenda for malaria elimination. The NMCP and malaria vector control stakeholders should benefit from the results of this review and devise an insecticide resistance management plan as part of their vector control activities. Systematic monitoring of resistance to antimalarial medicines and insecticides, and studies on malaria vector species composition, resting and biting behaviour has to be strengthened. All results on entomological monitoring surveys conducted in any region of Zimbabwe have to be rapidly and widely disseminated to pertinent government health staff, WHO and other relevant stakeholders in the field of malaria prevention and control. It is important to closely monitor outdoor transmission of malaria and the selection of malaria intervention strategies and their implementation in Mutare and Mutasa Districts should always be evidence-based.
CHAPTER 9

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

9.1 General discussion

This study examined the bionomics of malaria vector mosquitoes in Mutare and Mutasa Districts of Manicaland Province, Zimbabwe. Specifically, the study investigated species composition and abundance, resting and biting habits, as well as insecticide susceptibility levels in malaria vectors. Although a number of studies have been conducted on malaria vector mosquitoes in Zimbabwe within the last three decades (e.g. Mpofu, 1985; Taylor and Mutambu, 1986; Masendu et al., 2005), none examined malaria vector bionomics in Mutare and Mutasa Districts despite malaria being a disease of major public health importance in the two areas. Most of the studies on malaria vector bionomics have been conducted along the Zambezi Valley, Gokwe, Binga and Chiredzi Districts. Current information on bionomics of malaria vector species is important for purposes of designing the most appropriate malaria control interventions.

The ecological factors in Mutare and Mutasa Districts were found to be suitable for high breeding of malaria vector mosquitoes and the majority of mosquito larval habits in both sites are human-made, suggesting that transmission of malaria in the study areas is a result of various changes in the ecosystem for agricultural purposes by residents as was reported elsewhere in Africa (Ijumba and Lindsay, 2001). While it is essential to irrigate crops to improve food security and economic welfare, there is strong need to employ horticultural activities concurrently with selected larval source management to control the breeding of vector mosquito species (WHO, 2013b).

The present work demonstrated the occurrence of An. gambiae s.l. and An. funestus group in the study sites of Burma Valley and Zindi in Mutare and Mutasa Districts respectively. The two anopheline mosquito complexes were found breeding in sympatry and in different proportions. Members of the An. funestus group were more abundant than members of An. gambiae s.l. In contrast, previous work in Zimbabwe demonstrated that members of An. gambiae s.l. were the most abundant malaria vector species (Mpofu, 1985; Taylor and Mutambu 1986; Masendu et al.,
Members of the two complexes are important vectors of malaria in sub-Saharan Africa including Zimbabwe (Mpofu, 1985; Taylor and Mutambu 1986; Coetzee et al., 2000; Masendu et al., 2005). Masendu et al. (2005) reported four members of the *An. gambiae* complex in Zimbabwe, and these included *An. gambiae*, *An. arabiensis*, *An. merus* and *An. quadriannulatus* in sympatric occurrence. Of these four members, *An. arabiensis*, which is commonly found in association with the non-malaria vector *An. quadriannulatus*, was confirmed to be the major malaria vector in Zimbabwe (Masendu et al., 2005; Munhenga, 2010).

Elsewhere in Africa, sympatric occurrence of members of the *An. gambiae* complex has been reported (Coluzzi et al., 1979; Coetzee et al., 2000). The present study observed sympatric existence of *An. arabiensis* and *An. pretoriensis* (zoophilic and non-malaria vector) and these results are consistent with work by Masendu et al. (2005) in Zimbabwe which revealed coexistence of the two species, with dominance of *An. pretoriensis* over other *Anopheles* mosquitoes as observed from reared larvae.

Previous studies on the *An. funestus* group in Zimbabwe by Evans and Leeson (1935), showed sympatry of *An. funestus*, *An. leesoni* and *An. confusus*, whereas Green and Hunt (1980) reported *An. funestus*, *An. parensis* and *An. aruni* in sympathy in various parts of the country. This study demonstrated the occurrence of *An. funestus* and *An. leesoni* sibling species. In the *An. funestus* group, *An. funestus* is the only member that is implicated as an important vector of malaria in sub-Saharan Africa (Coetzee et al., 2000), while *An. rivulorum*, *An. leesoni* and *An. parensis* have been reported to be associated with minor transmission of malaria in the sub-region. Although *An. leesoni* was observed in the present study, its role in the transmission of malaria in Mutare and Mutasa Districts was not investigated.

Historically, *An. arabiensis* has been known to be the primary malaria vector in Zimbabwe while *An. gambiae* and *An. funestus* were secondary vectors of malaria (Mpofu, 1985; Masendu et al., 2005). A countrywide survey on malaria vector distribution in Zimbabwe in 2005 observed the occurrence of *An. funestus* only at Buffalo Ranch in Chiredzi District, Masvingo Province (Masendu et al., 2005). The low occurrence of *An. funestus* in Zimbabwe was attributed to its elimination following decades of IRS implementation. Interestingly, this study has shown the resurgence of *An. funestus* in Mutare and Mutasa Districts, demonstrating a shift in dominance of
Anopheles funestus from a secondary to a primary vector, with An. arabiensis being relegated to a secondary vector in the two districts.

The reason for the sharp rise in the An. funestus population in Mutare and Mutasa Districts where IRS has consistently been implemented over a decade were not clear. Possible reasons could be that pyrethroid-resistant An. funestus species have always continued to survive while densities of pyrethroid-susceptible An. gambiae and An. arabiensis dwindled following the prolonged use of pyrethroid-based IRS and LLINs against vector mosquitoes. Presumably, without nationwide studies on species composition and distribution in Zimbabwe, the recurrence of An. funestus populations observed in Mutare and Mutasa Districts may be illustrating a trend which is widespread in the country.

Species replacement in any setting as a result of vector control interventions is not a new phenomenon. Species shifts were reported in British Guiana following house-spraying using DDT (Giglioli, 1951). The study showed that the major malaria vector, An. darling, was eliminated and replaced by zoophilic An. aquasalis. A similar shift of species composition was reported during the inception of a large-scale house-spraying programme in East Africa where An. funestus was replaced with An. rivulorum following IRS with dieldrin in the 1950s (Gillies and Smith, 1960). The sympatric occurrence of malaria vector species and supremacy of An. funestus as demonstrated by the present work is a cause for concern for malaria control interventions. The findings are important in vector control programmes because An. funestus is known to be a more efficient malaria vector than An. arabiensis. Low densities of An. funestus have the potential to cause high levels of malaria transmission (Morgan et al., 2010). Additionally, in regions where breeding sites depend on rainfall patterns (examples being Mutare and Mutasa Districts); An. funestus prolongs malaria transmission during the dry season when the densities of An. gambiae and/or An. arabiensis have naturally declined (Fontenille et al., 1997; Dia et al., 2003).

Anopheles arabiensis and An. funestus observed in the present study may be associated with most malaria transmission in the villages surrounding the study sites. To control malaria transmission in Mutare and Mutasa Districts, Zimbabwe’s NMCP primarily employs vector control interventions, especially use of IRS and LLINs. Indoor residual spraying and LLINs have
been proved to be effective in reducing malaria transmission in most settings (Lengeler, 2004; Pluess et al., 2010; Kim et al., 2012). However, effective house-spraying or use of LLINs against malaria vectors, largely depends on whether mosquitoes rest or bite indoors (endophilic or endophagic), and this varies as it is affected by insecticidal irritancy or repellency (Pates and Curtis, 2005). This study confirmed that *An. funestus* has the tendency to rest indoors before or after feeding, a behavioural habit referred to as endophily. This behaviour was also shown in *An. gambiae s.l.* by Masendu (1996) in Gokwe and Binga Districts, Zimbabwe, in *An. funestus* by Mahande et al. (2007) in Tanzania, and in the *An. funestus* group and *An. gambiae s.l.* by Oyewole et al. (2007) in Nigeria.

The endophily rate for *An. funestus* was 84%, and this observation was confirmed by EWTs with gravid to feed index of constantly more than 1.0. If the ratio of gravid to feed is consistently above 1.0 the species has endophilic habits, and if the ratio is below 1.0 the mosquitoes have exophilic behaviour (WHO, 1975). However, the exophily rate of 16% observed in this study was lower than that observed by Dandalo (2007) in *An. gambiae s.l.* and *An. merus* in Gokwe District. The comparatively small proportion of exophilic tendencies demonstrated by *An. funestus* in this study should be taken into consideration when planning control strategies.

While *An. funestus* populations have been shown to be naturally endophilic in Africa (Pates and Curtis, 2005), the relatively small proportion of exophilic behaviour observed in this study could not be well understood. Presumably, the exophilic traits are associated with behavioural resistance which has evolved in *An. funestus* populations due to prolonged spraying programmes against malaria vector mosquitoes. Elsewhere in Africa, behavioural resistance in malaria vectors has arisen in response to prolonged spraying programmes (Pates and Curtis, 2005). This can have an impact on control efforts and may result from an immediate response to the irritant or repellent insecticides, especially DDT and pyrethroids. Indoor residual spraying can be effective only if the vector mosquitoes rest on the insecticide-treated surfaces for a sufficient time for them to pick up a lethal dose (Pates and Curtis, 2005).

Two categories of resting habitats for *Anopheles* mosquitoes were observed in this study and these included sprayable surfaces (walls and roofs/ceiling) and unsprayable surfaces (household furniture and other objects). Most of the mosquitoes were collected resting on sprayable surfaces.
Of those caught on the sprayable surfaces, roofs were the most preferred resting habitats than walls. In most structures, roofs have not been sprayed for the past five years in the study sites due to failure by the Zimbabwe’s NMCP to supply extension lances to reach heights of more than 3.5 m. Failure to spray roofs where the majority of An. funestus preferred to rest indoors has serious implications as it makes the species less vulnerable to IRS. However, the observed preference to rest on the roof than wall surfaces by most An. funestus mosquitoes caught could either be due to random chance or due to intense selection pressure leading to species tending to rest on unsprayed roofs/ceilings instead of sprayed walls during most of their movement rhythms.

Most adult An. funestus mosquitoes from the wall were collected at a height of 1 m from the ground. These results are consistent with observations by Perich et al. (2000) in Panama and Chadee (2013) in Trinidad. However, this is in contrast with observations by Harbison et al. (2006) in Kenya where a largest number of mosquitoes rested at heights of less than 0.8 m. For mosquito species that prefer to rest indoors, it is generally thought that the species would prefer to rest in dark, lower heights because they would be further away from the main door light source and the eaves, yet close enough to positions often occupied by humans (Bidlingmayer, 1994). Although the results of the current study are consistent with those reported by Bidlingmayer (1994), there is a potentially decreased efficacy of spray against vector mosquitoes because less insecticide dosages reach these lower heights. To confirm the low insecticide dosages on lower wall heights, Marevangepo (personal communication) reported WHO cone bioassay mortality of 34% in An. gambiae s.l. on sprayed wall of height less than 0.5 m from the ground, whereas a wall height of more than 1 m of the same structure had 100% mortality in Mutasa Districts. In Mpumalanga Province, South Africa, Govere et al. (2001) evaluated quality of spraying using WHO cone bioassay and 100% mortality in An. arabiensis was observed in all cones placed on the bottom, middle and upper positions of each deltamethrin-treated wall. Achievement of even deposition of insecticides on the surfaces largely depends on the ability and diligence of the spray operator to deliver the correct dose in the right location (Knapp et al., 2015).

It appears no studies have been conducted in Zimbabwe on tendencies by vector mosquitoes to rest on different surfaces and heights indoors. This information is critical for effective planning
and implementation of residual house-spraying, especially when complemented with observations on the tendencies by vector mosquitoes to exit sprayed or unsprayed houses. Information on behaviour of *Anopheles* mosquitoes to exit sprayed or unsprayed houses is crucial in determining species movement from inside to outside and the extent of irritability and repellency, as well as effect on species populations exiting treated structures (WHO, 1975).

Exit window traps fixed on both pyrethroid-sprayed and unsprayed structures collected mosquitoes in all four stages of blood digestion (unfed, fed, half gravid, gravid), with more *An. funestus* species being collected from unsprayed than sprayed structures. Interestingly, of those collected from structures recently sprayed using pyrethroids, the majority (more than 70%) of *An. funestus* were gravid in both study sites, with a 24-hour mortality of approximately 6%. The gravid mosquitoes collected from exit traps on structures recently treated using pyrethroids and low 24-hour mortality observed in this study is a cause for concern. These results suggest possible pyrethroid resistance to this malaria vector species and this requires further investigation.

Collections of unfed or freshly blood-fed females in exit traps on sprayed houses could most probably be associated with insecticide irritancy or repellency. Unfed females caught from exit traps set on unsprayed structures may suggest that the mosquitoes were most probably denied feeding by host avoidance behaviour. The 24-hour mortalities in *An. funestus* from exit traps set on unsprayed structures could be explained by the restrictive environment and unpleasant atmosphere created by the traps with efforts to escape or fly out that produced a stressful environment which in turn could have led to mortalities of mosquitoes from untreated structures.

To reduce mosquito bites, and finally malaria, Zimbabwe’s NMCP deployed LLINs to Mutare and Mutasa Districts. Although LLINs have been reported to be an efficient strategy to control malaria, their optimum effectiveness largely depends on vectors biting indoors and during hours when most people are in bed (Pates and Curtis, 2005). Although HLCs is the gold standard for measuring biting behaviour of vector mosquitoes, studies by Lines *et al.* (1991) in Tanzania and Fornadel *et al.* (2010) in Zambia showed that CDC light trap is comparable to HLCs, hence CDC light trap is considered a proxy tool for sampling malaria vector mosquitoes that would otherwise feed on humans. Fornadel *et al.* (2010) reported that in regions where use of vector control
interventions is high and vector densities are low, CDC light traps can be used to monitor vector HBRs, especially An. arabiensis. The numbers of mosquitoes caught by CDC light traps in the present work compare with those reported in similar studies (Lines et al., 1991; Davis et al., 1995) and that each trap was set next to a human and that the majority of the mosquitoes collected were unfed, suggesting that the mosquitoes were caught in the act of host-seeking.

Examining host-seeking behaviour, flight activity rhythms, host preferences and presence of sporozoites in Anopheles vector mosquitoes is critical in malaria control. Generally, the host-seeking activity of mosquitoes is not uniform; instead most species show distinct biting rhythm characteristic for each species (Pandian and Senthilkuamr, 2007). In the current study, flight activities of An. funestus and An. gambiae s.l. were more abundant indoors (68%) than outdoors (32%). This may suggest that the behaviour to seek hosts at night by An. funestus and An. gambiae s.l. could be interrupted by the use of LLINs, especially when most people in the study areas employ the tools every night all year round. Outdoor biting habits of vector mosquitoes mostly depend on the coincidence between intensity of biting outdoors and outdoor activities by the majority of people (Kabbale et al., 2013), and outdoor bites may be reduced by use of protective clothing and repellents.

While the biting habits of anopheline species are crucial for effective implementation of LLINs against malaria vector mosquitoes, it seems there are no previous studies in Zimbabwe that have examined indoor and outdoor biting behaviour of vector mosquitoes. The previous studies elsewhere in Africa and beyond that have examined malaria vector mosquitoes have identified variations in the biting behaviour. In Papua New Guinea, Kenya and Tanzania, a shift to outdoor biting was observed following widespread mosquito net use (Takken, 2002). Results of the present study are consistent with the study in Kamuli District, Uganda, that reported more indoor biting than outdoor biting catches for An. funestus (Kabbale et al., 2013).

Although the current work observed more indoor CDC light trap catches of An. funestus and An. gambiae complex than outdoors, the densities depended mainly on season. Anopheles funestus populations and An. gambiae s.l. were caught more abundantly in wet than dry season. These results are consistent with those reported by Oyewole et al. (2007) in Nigeria. In contrast,
Kabbale et al. (2013) in Uganda reported that both *An. gambiae s.l.* and *An. funestus* mosquitoes thrived all year round (wet and dry season) regardless of rainfall patterns.

The explanation for the variation in mosquito seasonal abundance in this study could be that in the wet season there would be abundance of larval habitats, suitable temperatures and relative humidity. These are the conditions most appropriate for breeding of mosquito species. Further, the current work observed the persistent occurrence of *An. funestus* populations from late wet season to early dry season and complete absence in the mid-dry season which coincided with winter, and a gradual increase from late dry season into early wet season. The abundance of *An. funestus* in the dry season could be attributed to the presence of more permanent water bodies for breeding provided by larger rivers, swamps, wells and irrigation channels in the two localities. Although the results of the current study showed abundantly more CDC light catches indoors in the wet season for both mosquito complexes, it remains crucial to maintain LLINs distribution campaigns all year round, scaling-up net hang-up campaign in wet season.

The use of LLINs against malaria would be optimized when most bites from malaria vectors occur during hours of the night when most people are in bed. In both study areas, *An. funestus* demonstrated variable nocturnal indoor and outdoor flight activity rhythms, with two peaks during the night; between 22:00-23:00 and 02:00-04:00 hours. In Gokwe District, Dandalo (2007) recorded biting peak at 22:00 hours in *An. gambiae s.l.* Elsewhere in Africa, Oyewole et al. (2007) in Nigeria observed two peaks which were different between species, with peak biting activities of *An. gambiae* indoor and outdoor at 22:00-01:30 hours and that for *An. funestus* at 20:00-03:00 hours. The results of this study showed that people who go to bed late after 23:00 hours or who wake up early before dawn for trade or agricultural activities are at risk of mosquito bites, contracting malaria as they get exposed to one of the peak cycles. To prevent mosquito bites during the two peaks for the people who would not be in bed for some reasons, it is important to use protective clothing and/or repellents. However, it could not be established why there was complete cessation of mosquito activities between 12 midnight and 01:00 hours in this study.

The results of multiplex PCR assay showed that the larger proportion of blood meals was human in origin, while a lesser percentage originated from domestic animals which included bovine,
dogs, goats and pigs. The overall HBI calculated from this study showed that more than 64% of blood meal originated from a human host, a fair proportion from domestic animals, while the least proportion comprised multiple blood meals (0.4%). In this study, multiple blood meals were lower than recorded in other studies that reported approximately 10-40% of blood-fed field specimens that contained multiple blood meals (Boreham et al., 1979; Wekesa et al., 1995). Results showed that about 35% of blood meals originated from domestic animals. The presence of domestic animal blood meals in An. funestus suggests that at least some individual An. funestus species may feed on domestic animals in the absence of human host, allowing for an opportunity to implement a zooprophylaxis malaria intervention strategy to complement the current tools.

The natural infection results showed that eight female An. funestus were found infected with P. falciparum, constituting a sporozoite rate of about 2% in both study sites. The sporozoite rates recorded in this study were relatively lower compared to the previous records in Zimbabwe (Evans and Leeson, 1935), but were close to those reported elsewhere in Africa (Bockarie et al., 1994; Oyewole et al., 2007). The detection of infection by Plasmodium species in female Anopheles mosquitoes suggests that An. funestus may be responsible for transmission of malaria in the villages within the two study sites. Moreover, it has been demonstrated by the results of this study that An. funestus is the primary malaria vector in the two sites, given its high densities, strong association with humans and its natural infection with Plasmodium parasites.

Since An. funestus is known for its highly anthropophilic, endophagic and endophilic behaviour (feeding on humans indoors and resting indoors) (Pates and Curtis, 2005), the use of LLINs and IRS may be the appropriate vector control interventions to reduce malaria transmission in the two study areas. However, these two vector control interventions are insecticide-based strategies that primarily depend on insecticide susceptibility status in the vector mosquitoes. The status of susceptibility to lambda-cyhalothrin, deltamethrin, etofenprox, bendiocarb, DDT and pirimiphos-methyl was evaluated in An. funestus wild populations and F1 progeny females collected from Burma Valley and Zindi sentinel sites following WHO (2013a) protocols for characterizing insecticide resistance.
This study was facilitated by the egg-laying method to generate sufficient number of *An. funestus* F1 progeny females. To achieve sufficient numbers of *An. funestus* females from larval collections is generally difficult and therefore production of F1 progeny females mostly relies on collecting live wild blood-fed or gravid indoor resting females for oviposition. Samples of wild caught *An. funestus* and F1 progeny females originating from both collection localities showed resistance to pyrethroids and carbamates, but were susceptible to DDT (OC) and pirimiphos-methyl (OP). While it is the first case of multiple resistances in a population of *An. funestus* from the two study sites, it is not the first in Africa. Such multiple resistances had already been observed in *An. funestus* from Mozambique with pyrethroid and carbamate resistance (Casimiro *et al*., 2006), from Ghana with pyrethroid, DDT and carbamate resistance (Okoye *et al*., 2008), and from Zimbabwe with pyrethroid and carbamate resistance (Choi *et al*., 2014).

The detected high level of pyrethroid and carbamate resistance in *An. funestus* mosquitoes reported for these two sites for the first time, and in neighbouring countries including South Africa (Hargreaves *et al*., 2000), Mozambique (Casimiro *et al*., 2006; Abilio *et al*., 2011), Malawi (Hunt *et al*., 2010), and Zambia (Chanda *et al*., 2011) is likely to become operationally significant and should be closely monitored. This is important since already there are reports of insecticide resistance in malaria vector mosquitoes in other parts of the country (Masendu *et al*., 2005; Munhenga *et al*., 2008; Choi *et al*., 2014). The lack of cross resistance between DDT and pyrethroids is an opportunity to the NMCP’s house-spraying operations.

It has been suggested that a cross-resistance mechanism was acting in the study sites to confer resistance to pyrethroids and carbamates through P450 monooxygenases (Brooke *et al*., 2001). Cross resistance between DDT and pyrethroids is often mainly conferred by *kdr* mutations (Martinez-Torres *et al*., 1998; Ranson *et al*., 2000; Coetzee and Koekemoer, 2013); hence it is assumed here that pyrethroid resistance is conferred by monooxygenases (P450) as there was no resistance mechanisms evaluated in this study. Pyrethroid resistance in *An. funestus* mosquitoes observed here is critical to the Zimbabwe NMCP as deltamethrin and lambda-cyhalothrin have been interchangeably employed for house-spraying against malaria vectors in Mutare and Mutasa Districts.
In addition to mortality, knockdown time is an important indicator for the early detection of reduced susceptibility in vector populations. Knockdown time has been accepted as an indicator of susceptibility (Kang et al., 1995). Therefore, the measurement of knockdown time could systematically be included in insecticide resistance monitoring programmes of mosquitoes since this time provides preliminary information on the possible involvement of the kdr gene (Chandre et al., 1999).

The KD$_{50}$ and KD$_{95}$ for all insecticides assayed in An. funestus mosquitoes collected from both sites were relatively high. The increase in knockdown time was more marked with pyrethroids and carbamates than DDT and pirimiphos-methyl. While DDT and pirimiphos-methyl resistance was not detected in this study, the levels of KD$_{50}$ and KD$_{95}$ for the two insecticides were comparatively higher than those reported in Kamhororo, Masakadza and Chilonga villages in Zimbabwe (Lukwa et al., 2012). Results of this work may suggest that a knockdown resistance mechanism could be operating at lower level in this mosquito population, and if not closely monitored could be a threat to future house-spraying.

The level of resistance has possibly increased by prolonged house-spraying, agricultural and domestic use of pyrethroids. However, the main use of pyrethroids in the study areas is not for malaria control, but for agricultural purposes, and to a lesser extent, for control of domestic pests. The wide application of insecticides, especially in agriculture has been implicated in an increase in the selection pressure for resistance in Anopheles mosquitoes (Chandre et al., 1999) and therefore the selection pressure is difficult to control. It seems there are no documented records of carbamate use for IRS in the study sites, and most probably, the observed carbamate resistance may have resulted from its widespread use in agriculture.

To gather general information on the strength of the insecticide resistance recorded here, resistance intensity assays were included. The resistance intensity tests carried out here were not a standard method for measuring resistance, but the results are essential as they provide preliminary information on the level of insecticide resistance which can assist to plan for malaria control. At the 8-hour interval, An. funestus populations showed a knockdown rate of less than 90% on the WHO diagnostic dose of 0.05% lambda-cyhalothrin and 0.05% deltamethrin in both sites, indicating a relatively high level of resistance.
It is evident from the susceptibility studies that an insecticide resistance management and monitoring strategy needs to be developed and implemented in order to reduce malaria transmission in Mutare and Mutasa Districts. To manage the observed insecticide resistance, house-spraying using DDT or organophosphates or pyrethroid-treated LLINs with a synergist could be employed. Deployment of pyrethroid-treated LLINs or carbamate-based IRS is regrettably not an option with such a high mosquito survival rate. The *An. funestus* group is susceptible to pirimiphos-methyl and DDT, raising the possibility of using organophosphates for house-spraying against vector mosquitoes, may be in rotation with DDT. It is also important to strengthen basic research on resistance mechanisms and operational research on the impact of resistance on the efficacy of IRS and LLINs.

### 9.2 General conclusion

This study provided strong evidence that *An. funestus* is the primary malaria vector in the study sites, and the species is resistant to pyrethroids and carbamates, but susceptible to DDT (OC) and pirimiphos-methyl (OP). Moreover, it was clear that *An. funestus* occurs in sympatry with *An. arabiensis*, with the former preferring to breed in various habitats created by the farmers for agricultural purposes. *Anopheles funestus* populations and *An. gambiae s.l.* were more endophilic than exophilic and CDC light traps collected the two complexes more abundantly indoors than outdoors. Transmission of *P. falciparum* is maintained primarily by *An. funestus* with *An. arabiensis* contributing secondarily, in the apparent absence of *An. gambiae*. Due to its resistance to pyrethroids and carbamates observed here, efficiency to transmit malaria, as well as poor adaptability to laboratory conditions, *An. funestus* species represent a serious threat to the inhabitants of Burma Valley and Zindi.

Considering that pyrethroid and DDT resistance in *An. arabiensis* mosquitoes had previously been reported in Zimbabwe only from Gwave village in Gokwe District in 2005 and 2008 and pyrethroid and carbamate resistance in *An. funestus* only in Mandeya village in Mutasa District in 2014, it is therefore worrying to detect pyrethroid and carbamate resistance at such high levels in a village in Mutasa District, and a village in Mutare District which are separated by a distance of about 200 km. It is possible that the resistance could be more wide spread than previously
thought. Whatever the cause, the implications of this resistance scenario for insecticide-based malaria control are severe and require urgent attention.

With no new classes of insecticides for malaria control on the market, malaria programme managers have few options available when threatened with multiple insecticide resistance. The resistance levels recorded in this report, combined with continual insecticide selection pressure will inevitably reduce the efficacy of IRS and LLINs against malaria vector mosquitoes. If it is not closely monitored and effectively managed, this resistance could spread rapidly to other districts and provinces and threaten to reverse the milestones that have been made in reducing malaria transmission in Zimbabwe.

9.3 Recommendations

i. NMCP must change the current insecticides being employed for IRS from pyrethroids to organophosphorus products or DDT (organochlorine). Thereafter, a rotation of organophosphates with organochlorines (DDT) or mosaic use of the two classes of insecticides observed here to be effective against the major vector An. funestus is important to manage insecticide resistance in future years.

ii. It is critical to come up with an effective insecticide resistance management and monitoring plan for malaria vectors. The strategy will be most helpful for managers of NMCPs, MOHCC vector control staff and various agencies involved in planning and implementing malaria vector control strategies.

iii. NMCP is recommended to provide extension lances to spraying programme to enable spraying of higher roofs/ceilings as these were observed to be the most preferred indoor resting places of mosquitoes.

iv. There is need to use measures such as protective clothing and mosquito repellents to complement LLINs, especially if residents are not in bed during peak mosquito densities.

v. Selective larval source management must be implemented as a supplementary measure to the current vector control strategies.
vi. Further studies are required to examine the occurrence and distribution of *An. funestus* mosquitoes in Manicaland Province, and elucidate all possible resistance mechanisms that might be contributing to insecticide resistance.
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ANNEX 1

MALARIA VECTOR SPECIES COMPOSITION AND RELATIVE ABUNDANCE IN MUTARE AND MUTASA DISTRICTS, ZIMBABWE

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**Key words:** Abundance, anopheline, mosquito, species.

**Authors’ contributions:** SS, conception of the problem, design, collection, PCR assays, analysis, interpretation and drafting of final article; MZ, PC and HTM, responsible for all the stages of the research, including analysis and interpretation as well as critically reviewing the final draft for academic worth; AM, data collection, PCR assays, analysis, interpretation and drafting of final article.

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**Conference presentation:** this paper was presented at Zimbabwe National Malaria Conference, 2014 September 1-4, Kadoma, Zimbabwe.
Abstract

Regular entomological monitoring is important to determine changes in mosquito species composition and relative densities of malaria vectors in relation to vector control interventions. A study to gain insights into malaria vector species composition and relative abundance was undertaken in Mutare and Mutasa districts, Zimbabwe. Two methods; indoor resting catches and larval sampling were used to collect indoor resting adults and larvae from May 2013 to April 2014. Mosquitoes collected as adults and reared from larvae that were identified morphologically as potential malaria vectors were further processed to sibling species by polymerase chain reaction (PCR). Morphological identification of anopheline mosquitoes showed presence of two complexes: *An. funestus* and *An. gambiae*. The total number of female members of the *An. funestus* group and *An. gambiae* complex collected by both methods from the two sites was 840 and 31 respectively. Malaria vector species of both complexes were more abundant in Mutare than in Mutasa. The PCR-based assays showed the presence of four sibling species: *An. funestus sensu stricto* (90.8%, 267/294) and *An. leesoni* (5.1%, 15/294), of *An. funestus* group; *An. arabiensis* (41.9%, 13/31) and *An. quadriannulatus* (48.4%, 15/31) of the *An. gambiae* complex. About 4% and 5% of specimens of *An. gambiae* complex and *An. funestus* group respectively did not amplify. Of the two identified malaria vector sibling species, *An. funestus sensu stricto* was more abundant (95.4%, 267/280) than *An. arabiensis* (4.6%, 13/280), suggesting the replacement to secondary vector of *An. arabiensis* which was previously the predominant vector species. *An. funestus sensu stricto* and *An. arabiensis*, the most important vectors of human malaria were identified in this study, but their resting and biting habits as well as insecticide susceptibility are unclear. Further studies on vector behaviour are therefore recommended.

Introduction

Malaria in Zimbabwe is a serious public health problem, causing morbidity, mortality and poverty although control efforts aimed at the vector mosquito are set up annually (Lukwa et al., 2014). The most important vector species of human malaria in Africa belong to *An. gambiae* complex and *An. funestus* group. In Zimbabwe, malaria is transmitted by vector species

Previous studies on *An. gambiae* complex in Zimbabwe documented four members; *An. gambiae*, *An. arabiensis*, *An. merus* and *An. quadriannulatus* in various combination of sympatry (Masendu et al., 2005). The wide distribution of *An. arabiensis*, often in association with the non-vector *An. quadriannulatus*, confirmed its status as the principal human malaria vector in Zimbabwe (Masendu et al., 2005; Munhenga, 2010).

Historically, the *An. funestus* group consists of at least nine African species: *An. funestus sensu stricto* (hereafter referred to as *An. funestus*), *An. rivulorum*, *An. vaneedeni*, *An. leesoni*, *An. confusus*, *An. fuscivenosus*, *An. brucei*, *An. parensis* and *An. aruni* (Gillies and de Meillon, 1968; Gillies and Coetzee, 1987). Recently, new sibling species including *An. rivulorum-like* (from West Africa), *An. funestus-like* (from Malawi) and *An. funestus-like-like* (from Zambia) (Spillings et al., 2009) have been isolated.

In the *An. funestus* group, *An. funestus* is the only member that is implicated as an important vector of malaria in sub-Saharan Africa (Coetzee et al., 2000). The sympatric occurrence of *An. funestus* with minor vectors *An. rivulorum* and *An. leesoni* has been found in several countries in Africa (Wilkes et al., 1996; Cohuet et al., 2003) but has not been reported in Zimbabwe.

The importance of the above vectors in malaria transmission differs depending on their feeding and resting preference behaviour, seasonal abundance and vectorial capacity (Coluzzi, 1984). These differences therefore, contribute to the varied malaria epidemiological patterns observed in Africa and, subsequently, different vector behaviour may require different strategies for optimal vector control.
Currently, the two most common vector control strategies are indoor residual spraying (IRS) and long lasting insecticidal nets (LLINs), accounting for almost 60% of global investments in malaria control (WHO, 2013). Molineaux and Gramiccia, (1980) concluded that differences in vector behaviour and insecticide resistance cause the failure of IRS and/or mosquito nets in suppressing malaria transmission in any setting. As such, different vector species may have different insecticide susceptibility status at any given locality and time, thus, insecticides for vector control need to be selected carefully to achieve maximum impact on malaria transmission.

Current vector control studies in Zimbabwe, especially on species composition and relative abundance in relation to malaria control focus mainly on members of the An. gambiae complex. This focus on only An. gambiae complex and marginalizing the An. funestus group may be due to fairly easy larval collection and adaptability of the An. gambiae complex to field insectary and laboratory conditions as well as the scarcity of members of the An. funestus group. The scarcity of An. funestus in Zimbabwe is believed to be associated with consistent implementation of IRS which commenced in the early 1950s and expanded in 1980s following national independence in 1980 and scaled up in the mid-1990s (Munhenga, 2010).

An. funestus was last reported about ten years ago by Masendu et al. (2005) only at Buffalo Ranch in Chiredzi district of Masvingo province in the southern region of Zimbabwe. More recently, it was reported in Honde Valley, Zimbabwe (Choi et al., 2014). Despite continued IRS programmes in place, there is a real possibility of An. funestus resurgence as the focus of entomological studies has been almost exclusively on the An. gambiae complex. Consequently, the current malaria species composition and densities, especially of An. funestus remain unknown in the study areas.

Up-to-date information on species composition and densities of both primary and secondary vectors is crucially needed to properly devise and implement vector control activities to prevent malaria transmission and to assess their effectiveness. Therefore, this study aimed to determine malaria vector species composition and relative abundance in Burma Valley and Zindi in Mutare and Mutasa districts, respectively.
Materials and methods

Study areas

The study was undertaken in Burma Valley administrative ward (19°11’S, 32°48’E), Mutare district, and Zindi administrative ward (18°22’S, 32°56’E), Mutasa district of Manicaland province, Zimbabwe (Figure 1). Studies were done from May 2013 to April 2014. The sites are situated in rural areas with similar ecological settings. The ecological features of the sites, however, show that they consist predominantly of tropical savanna, grassland and woodland ecosystem characterized by grass, trees and bushes, widely spaced and typically associated with the tropical wet and dry climate type.

Figure 1. Map of Manicaland province, Zimbabwe, showing the study sites
The climate is tropical with annual temperature, relative humidity and rainfall ranges of 18-30°C, 65-85% and 900-1200 mm, respectively (Taylor and Mutambu, 1986). Additionally, the climate presents three clearly different seasons: a cold-dry season between April and August, with little rains in April and at times extremely cold in July, hot and dry season in August through to October, completely without rains, and a hot-wet season stretching from November to March with a potential of high rainfall during the months of December and February (Taylor and Mutambu, 1986). Both sites are valleys (679 and 766 metres above sea level for Burma Valley and Zindi, respectively) with several streams and rivers that flow into Mozambique.

Majority of the community members are subsistence farmers who grow mainly maize, bananas and yams along the river banks. Income is derived from the sale of their agricultural produce in Mutare, Harare and Bulawayo urban markets. The farmers use mainly pyrethroid and organophosphate classes of insecticides to protect their crops against several agricultural pests.

Selection of study sites was based on the ecological conditions which are potential breeding sites for malaria vectors. Malaria transmission in the two study areas is a major public health problem and occurs seasonally, especially in November through to May with a peak in March/April (Lukwa et al., 2014). In June and July, the two sites experience the lowest malaria transmission (Lukwa et al., 2014).

Several strategies which included IRS, LLINs, larval source management, intermittent preventive treatment in pregnancy, diagnosis and case management as well as social behaviour change communication were put into action to control both the malaria vectors and parasites, to reduce malaria transmission in the study sites. Among these interventions, IRS, LLINs and malaria case management have been scaled-up to cover most villages of the study areas (Lukwa et al., 2014).

**Mosquito larval sampling and rearing**

Weekly physical examinations of natural and human-made mosquito larval habitats were conducted to determine the availability of potential breeding sites for both *An. gambiae sensu*
*lato* (s.l.) and *An. funestus* group for two months for five days per month from September to October 2013. Types of breeding sites were categorised into human-made and natural in origin and recorded. Larval collection was performed once a month from November 2013 to April 2014. Where mosquito larvae were present, 5-10 dips, depending on the size of the habitat, were taken using standard dippers of 350 ml capacity. Samples were classified into 1) group of 1\textsuperscript{st} and 2\textsuperscript{nd} instars, 2) group of 3\textsuperscript{rd} and 4\textsuperscript{th} of instars, and 3) pupae. The instars were identified morphologically using taxonomic keys of Gillies and De Meillon, (1968). The number of 1\textsuperscript{st} to 4\textsuperscript{th} instar larvae of each anopheline species collected per dip represented the larval density in each breeding site.

Immediately after morphological determination of species and larval density assessment, the *Anopheles* larvae were placed in plastic jugs and taken to entomological field laboratories/insectaries for rearing according to WHO, (2003) guidelines. All larvae were kept at room temperature and fed with ground fish food. The adults that emerged were transferred to the laboratory at National Institute of Health Research (NIHR) in Harare. The specimens were killed by anaesthetizing with drops of acetyl acetate placed on a large filter paper that was held above the adults’ container. Emerged adults were identified morphologically into species complexes (*An. gambiae* and *An. funestus*) using taxonomic keys of Gillies and Coetzee (1987). Afterwards, they were individually preserved on silica gel in well-labelled eppendorf tubes prior to polymerase chain reaction (PCR) assays. Other anophelines were morphologically identified (Gillies and Coetzee, 1987), recorded and discarded.

**Indoor collections**

Indoor resting adult mosquitoes were collected by the pyrethrum spray catch (PSC) method (WHO, 2003) in twenty conveniently selected bedrooms in Burma Valley and Zindi, ten bedrooms apiece for one day per month from May 2013 to April 2014. The selected bedrooms were visited between 6.30 and 10.00 am every study day to collect adult mosquitoes. Anophelines were sorted and identified morphologically into *An. gambiae s.l.* and *An. funestus* group as well as other anophelines (Gillies and Coetzee, 1987). Female specimens were
preserved in labelled eppendorf tubes containing silica gel as drying agent awaiting further processing at NIHR. Males were recorded and discarded.

**Polymerase chain reaction species identification**

Polymerase Chain Reaction (PCR) was carried out using deoxyribonucleic acid (DNA) extracted from two legs of each morphologically identified specimen. *Anopheles gambiae s.l.* specimens were PCR-assayed using a protocol described by Scott et al. (1993) and amplified using specific diagnostic primers for *An. gambiae*, *An. arabiensis* and *An. quadriannulatus*. As for *An. funestus* group, samples were assayed following the methods of Koekemoer et al. (2002) with minor modifications as detailed by Spillings et al. (2009) using primers for *An. funestus*, *An. leesoni*, *An. vaneedeni*, *An. parensis*, *An. rivulorum*, *An. rivulorum*-like and *An. funestus*-like. The results of PCR amplification were visualized on 1% agarose gel by ethidium bromide staining.

**Data analysis**

Data were analysed using analysis of variance (ANOVA) at 95% confidence limit. The relative abundance of the species was expressed as the percentage of the total number of *Anopheles* collected.

**Ethical considerations**

Confidentiality and voluntary participation was assured to the households members who were included for PSC. Signed informed consent form was obtained from each participant before collecting mosquitoes from the bedrooms.
Results

Larval habitat census

A total of 42 habitats positive for breeding of aquatic stages of mosquitoes were identified in the study areas (Figure 2). Rain pools were temporary, shallow wells, yam plantations and river banks were semi-permanent, while irrigation channels as well as marshes were permanent mosquito habitats. Of these breeding sites, 23 were in Burma Valley and 19 in Zindi. For both study sites, the overwhelming majority of the anopheline-positive habitats were human-made (88.1%, 37/42) with the remainder natural in origin (11.9%, 5/42). Chances of sampling anopheline larvae were higher in irrigation channels in Zindi, but highly heterogeneous in yam plantations in Burma Valley area. All larval habitats were located within 2 km of the homesteads of both study sites.
Figure 2. Types of mosquito breeding habitats in Burma Valley and Zindi, Mutare and Mutasa districts respectively, Zimbabwe: shallow well (A), yam plantation (B), irrigation channel (C), rain pool (D), marsh (E) and river bank (F).

**Larval habitat support and relative abundance**

Habitat support for larval development differed at the two study sites. In Burma Valley, 34.8% (8/23) of the habitats had only anopheline, while 17.4% (4/23) had only culicine, and were visited 12 times. In Zindi, 15.8% (3/19) of the habitats had anopheline only and 36.8% (7/19) had only culicine, and were visited 10 times. This gave a combined total of 22 longitudinal samples in 12 months for the two study areas. Sympatry in anopheline and culicine larvae was found in 47.8% (11/23) of the habitats in Burma Valley and 47.4% (9/19) in Zindi, suggesting that the mosquito larvae from the subfamilies *Anophelinae* and *Culicinae* coexist in almost half of the habitats.

A total of 3227 immature stages of anopheline larvae (2438 early instars, 789 late instar) were collected in Burma Valley and 1621 (872 early instars, 749 late instars) in Zindi. Pupae were observed in 28.6% (12/42) of larvae-positive sites. *Anopheles* larvae were more abundant in Burma Valley than in Zindi (ANOVA: df=6; F=12.11; P=0.00). The mean density of *Anopheles* larvae over the entire sampling efforts was 4.2 and 1.8 larvae per dip in Burma Valley and Zindi, respectively. The majority of *An. funestus* larvae were collected in irrigation channels, marshes and shallow wells, whilst *An. gambiae s.l.* larvae were found in rain pools, yam plantations and river banks.

**Species composition of anopheline larvae**

Of the approximately 4848 *Anopheles* larvae collected, a total of 4690 adult mosquitoes emerged. From these, two malaria species complexes were morphologically identified, namely, *An. funestus* group and *An. gambiae s.l.* The *An. funestus* group accounted for (1.9%, 87/4690) of the adults, while *An. gambiae s.l.* constituted (0.2%, 10/4690), with *An. pretoriensis*, a non-malaria vector species contributing the majority (97.9%, 4593/4690) in both study sites. Overall, the densities of adults of the *An. funestus* group and *An. gambiae s.l.* that emerged from the
larval collections were low, but the *An. funestus* group was about 8 times more abundant than *An. gambiae s.l.*

**Species composition and abundance of adult Anopheles mosquitoes**

A total of 5625 *Anopheles* adults, including males and females were collected from larval sampling and indoors through PSC methods (Table 1). Of the total collections, *An. pretoriensis* was the most abundant while the remainder was shared between the *An. funestus* group and *An. gambiae s.l.* Overall, more anopheline mosquitoes were collected from aquatic stages (i.e. reared from larvae) than indoors (i.e. as adults), but indoor collections by PSC method had more malaria vector mosquitoes than larval collection. Of note, the results showed that the combined female *An. funestus* group and *An. gambiae s.l.* were approximately 5 times more abundant than males (Table 1).

Table 1. Percentage mosquito species composition sampled in Burma Valley and Zindi as identified by morphological means (actual numbers in parentheses).

<table>
<thead>
<tr>
<th>Sampling method‡</th>
<th>Study area</th>
<th>N</th>
<th>An. funestus group</th>
<th>An. gambiae s.l.</th>
<th>An. pretoriensis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>PSC</td>
<td>Burma Valley</td>
<td>(795)</td>
<td>16.0</td>
<td>80.6</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>(140)</td>
<td>9.3</td>
<td>87.1</td>
<td>0.7</td>
</tr>
<tr>
<td>RFL</td>
<td>Burma Valley</td>
<td>(3141)</td>
<td>0.3</td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>(1549)</td>
<td>0.1</td>
<td>2.8</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>(5625)</td>
<td>2.6</td>
<td>14.9</td>
<td>0.2</td>
</tr>
</tbody>
</table>

‡PSC = Pyrethrum spray catch; RFL = Reared from larvae

**Species composition and abundance of female adult malaria vectors**

The species composition and the relative abundance of female malaria vector mosquitoes are presented in Table 2. Altogether, more than 800 female *An. funestus* group and *An. gambiae s.l.*
mosquitoes were collected. As observed from Table 2, there was no difference in species composition in the two sites (ANOVA: df=1; F=3.25; P=0.17), though the relative abundance of \textit{An. funestus} group and \textit{An. gambiae s.l.} was more in Burma Valley than in Zindi (ANOVA: df=9; F=3.65; P=0.00). All in all, members of the \textit{An. funestus} group were 27 times more abundant than \textit{An. gambiae s.l.} in both sites (Table 2).

<table>
<thead>
<tr>
<th>Sampling method‡</th>
<th>Study area</th>
<th>Total number</th>
<th>\textit{An. funestus} group</th>
<th>\textit{An. gambiae s.l.}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSC</td>
<td>Burma Valley</td>
<td>(663)</td>
<td>96.7 (641)</td>
<td>3.3 (22)</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>(126)</td>
<td>96.8 (122)</td>
<td>3.2 (4)</td>
</tr>
<tr>
<td>RFL</td>
<td>Burma Valley</td>
<td>(37)</td>
<td>91.9 (34)</td>
<td>8.1 (3)</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>(45)</td>
<td>95.6 (43)</td>
<td>4.4 (2)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>(871)</td>
<td>\textbf{96.4 (840)}</td>
<td>\textbf{3.6 (31)}</td>
</tr>
</tbody>
</table>

‡PSC = Pyrethrum spray catch; RFL = Reared from larvae

**PCR analysis of \textit{An. funestus} group sibling species**

From a total of 840 females that had been morphologically identified as members of the \textit{An. funestus} group, 294 specimens were PCR assayed. Two sibling species were identified: \textit{An. funestus} (90.8%, 267/294) at a position of 505 base pairs (bp) and \textit{An. leesoni} (5.1%, 15/294) at 146 bp (Figure 3). Twelve specimens (4.1%, 12/294) failed to amplify after two replicates, though the positive control amplified successfully.
Polymerase chain reaction based assays to identify sibling species of *An. gambiae* s.l.

Out of a total of 31 females positively identified to be *An. gambiae* s.l., 48.4% (15/31) were identified as *An. quadriannulatus* while 41.9% (13/31) were *An. arabiensis* (Figure 4). About 9.7% (3/31) of the *An. gambiae* s.l. tested could not be identified to specific species by PCR using the then available primers specific for *An. gambiae*, *An. arabiensis* and *An. quadriannulatus* sibling species.
Figure 4. Species identification of members of *An. gambiae* s.l. from Burma Valley and Zindi: *An. quadriannulatus* (150 base pair) (lanes 1, 3-8, 10, 12-14 and 16), *An. arabiensis* (315 base pair) (lanes 2, 11 and 15), 100 base pair molecular marker (lane 9).

**Percentage species composition and abundance of *An. funestus* and *An. arabiensis* in Burma Valley and Zindi**

*An. funestus* and *An. arabiensis* sibling species were present in both study sites, though both species were less abundant in Zindi than in Burma Valley (Table 3). The relative abundance of *An. arabiensis* was generally low in both sites. Overall, *An. funestus* was more abundant than *An. arabiensis* in both sites (ANOVA: df=2; F=14.20; P=0.02) (Table 3).

Table 3. Percentage of species composition and abundance of *An. funestus* and *An. arabiensis* in Burma Valley and Zindi areas (count data in parentheses).

<table>
<thead>
<tr>
<th>Study area</th>
<th>Total number</th>
<th><em>An. funestus</em></th>
<th><em>An. arabiensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Burma Valley</td>
<td>(169)</td>
<td>95.3 (161)</td>
<td>4.7 (8)</td>
</tr>
<tr>
<td>Zindi</td>
<td>(111)</td>
<td>95.5 (106)</td>
<td>4.5 (5)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>(280)</strong></td>
<td><strong>95.4 (267)</strong></td>
<td><strong>4.6 (13)</strong></td>
</tr>
</tbody>
</table>
Discussion

The study showed the presence of two *Anopheles* complexes: *An. gambiae* and *An. funestus* in Burma Valley and Zindi. Both complexes contain important malaria vectors in sub-Saharan Africa (Coetzee et al., 2000). Morphologically identified adults reared from larval collections revealed the presence of predominantly *An. pretoriensis* (a non-malaria vector) and relatively low numbers of adults of the *An. funestus* group and *An. gambiae s.l.* The members of the *An. funestus* group were, however, relatively more abundant than members of *An. gambiae s.l.* (ANOVA: df=4; F=4.44; P=0.04). These findings are consistent with previous studies in Zimbabwe that demonstrated dominance of *An. pretoriensis* reared from larvae over other anopheline species (Masendu et al., 2005).

This study clearly showed that malaria vectors belonging to the *An. funestus* group and *An. gambiae s.l.* breed in sympatry in the study sites. Most of the mosquito larval habitats in the study areas are human-made; suggesting that malaria transmission in the study areas is derived mainly from human modification and manipulation of the ecosystem. While the irrigation facilities are important to sustain food security, mitigating measures need to be put in place to minimize breeding of vector mosquitoes.

In the present study, members of the *An. funestus* group were more abundant than members of *An. gambiae s.l.* from both adult and larval collections. Previous work by Mpofu (1985), Taylor and Mutambu (1986) and Masendu et al. (2005) had shown members of *An. gambiae s.l.* to be the more abundant malaria vector in Zimbabwe.

Using PCR, the present study identified two species: *An. funestus* and *An. arabiensis* that transmit malaria in the study sites. PCR identification of sibling species of the *An. funestus* group and *An. gambiae s.l.* is of great importance to the malaria control programme in Zimbabwe. Molecular analysis of the *An. funestus* group established the presence of *An. funestus* and *An. leesoni*. This is in agreement with studies by Oyewole et al. (2005) but in contrast with other previous studies in Africa which recorded *An. funestus* in sympatry with minor vectors *An. rivulorum* and *An. leesoni* (Wilkes et al., 1996). The failure to amplify 5.1% of the *An. funestus*
group could be due to morphological misidentification of some specimens and/or emergence of other sibling species.

Meanwhile, PCR assay of *An. gambiae s.l.* also revealed two common members: *An. quadriannulatus* and *An. arabiensis*, but could not exclude the possibility that other members of the complex might be amongst the specimens (9.7%) that failed to amplify. It is likely that the unidentified *An. gambiae s.l.* could be *An. merus* as the protocol by Scott et al. (1993) used in this study did not include primers to identify *An. merus* which Masendu et al., (2005) reported as being common in some parts of Zimbabwe.

Polymerase chain reaction assays revealed *An. funestus* to be more abundant than *An. arabiensis*, confirming the status of the former as a major malaria vector mosquito in both sites. This is in sharp contrast with results from work by Mpofu (1985), Taylor and Mutambu (1986) and Masendu et al. (2005) which had shown *An. arabiensis* to be a primary vector while *An. gambiae* and *An. funestus* were secondary vectors in Zimbabwe. The sharp increase in *An. funestus* population in the presence of IRS implementation in this study sites cannot be explained in this study. It is more likely that pyrethroid-resistant *An. funestus* survived undetected while *An. arabiensis* and to a larger extent, *An. gambiae*, remained susceptible to pyrethroids which have been used for IRS and treatment of LLINs over the years in the study areas.

In general, species replacement for various reasons, especially as a result of IRS and LLINs is not a new phenomenon (Kitau et al., 2012). Recent data from East Africa showed changes in sibling species following the scaling-up of ITNs/LLINs, with *An. arabiensis* becoming the dominant species in habitats that previously supported sympatric *An. gambiae* and *An. arabiensis* populations (Kitau et al., 2012). A similar change in species composition and abundance was reported during the implementation of IRS in Zimbabwe; example being the observation of *An. funestus* in most parts of Zimbabwe by Mpofu, (1985), which was isolated in later studies by Masendu et al. (2005) only at Buffalo Ranch in Chiredzi district.

As revealed by the findings of this study, heterogeneity of vector species composition and dominance of *An. funestus* is of major significance in malaria control. The results have important
implications for malaria epidemiology and control given that *An. funestus* is a more efficient vector than *An. arabiensis*. Low densities of *An. funestus* have the potential to sharply increase levels of malaria transmission (Morgan et al., 2010).

This study also revealed that there is information gap on the resting, biting and insecticide susceptibility status of malaria vectors in the study sites. The information is crucial for planning, implementation and evaluating malaria vector control strategies. Additionally, the emergence/upsurge of *An. funestus* in areas under IRS and LLINs use requires urgent attention to prevent possible malaria outbreaks. Mitigation measures for irrigation projects or farming should be put in place to minimize malaria vector breeding.

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ANNEX 2

INSIGHTS INTO RESTING BEHAVIOUR OF MALARIA VECTOR MOSQUITOES IN MUTARE AND MUTASA DISTRICTS, ZIMBABWE

7 This chapter has been published as: Sande, S., Zimba, M., Chinwada, P., Masendu, H.T. and Makuwaza, A. 2016. Insights into resting behavior of malaria vector mosquitoes in Mutare and Mutasa districts of Manicaland province, Zimbabwe. doi: 10.1093/jme/tjw044.
Abstract

A study was conducted to investigate the current resting behaviour of malaria vectors in Mutare and Mutasa districts, Zimbabwe. Mosquitoes were captured using pyrethrum spray collection, prokopac aspirator, pit shelter, and exit trap methods. Mosquitoes were sorted and identified using morphological key and polymerase chain reaction (PCR) techniques. The *Anopheles funestus* group constituted 97% whereas *An. gambiae* complex were few (3%). Endophilic collections in both species were five times greater than exophilic catches. The endophilic trait was further demonstrated by gravid to feed index (gravid/fed) of constantly more than one. Nearly 90% endophilic *An. funestus* populations were collected on sprayable and 10% collected on unsprayable surfaces. Of the sprayable surfaces, 56% were collected on the roofs, with 44% on the walls. Of those on the walls, 44%, 22%, and 34% were caught on wall heights >1 m, 1.0-1.5, <1.5 m from the ground respectively. Of the gravid *An. funestus* caught, nearly two-thirds were collected exiting pyrethroid-treated structures, with a 24-hour mortality of less than 10%. The PCR analysis of 120 specimens taken randomly from the *An. funestus* group was all *An. funestus s.s.* The present work indicates that for effective malaria control in Mutare and Mutasa districts using indoor residual spraying, both walls and roofs must be sprayed.

Key words

Mosquitoes, resting behaviour, indoor, outdoor, *Anopheles funestus*

*Anopheles funestus* Giles is one of the most known malaria vector mosquitoes in sub-Saharan Africa (Gillies and De Meillon 1968). In addition, even in the presence of other efficient vector species like *An. gambiae s.s.* Giles and *An. arabiensis* Patton; *An. funestus s.s.* contributes a large proportion of infectious bites that maintain malaria transmission among different communities.

Malaria remains the most important vector-borne public health problem globally, with the major burden occurring in the African Region (WHO 2014, WHO 2015a, WHO and UNICEF 2015). In 2010, the World Health Organization (WHO) estimated that approximately 99 countries still had ongoing high burden of malaria annually, 219 million cases (range 154-289 million), with the
disease killing an estimated 660,000 people. Approximately, 81% of cases and 91% of the deaths occurred in the WHO African Region, 86% of the victims were children under five years of age (WHO 2015b). In Zimbabwe, malaria continues to be the major parasitic disease of public health importance causing morbidity and mortality despite intensive prevention and control efforts.

Vector control is currently the major intervention for global malaria prevention, control and elimination (WHO 2015a). It remains critical for the reduction in malaria incidence and deaths. Presently, indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) are the two major vector control strategies that contribute to the prevention and control of malaria transmission (WHO 2010). Indoor spraying of houses with residual insecticides reduces the longevity of indoor resting anopheline mosquitoes, greatly limiting the probability of malaria transmission. The effectiveness of IRS in combating malaria transmission and disease burden was first shown in the 1930s in South Africa (De Meillon 1936, Park 1936) and India (Covell et al. 1938), and late 1940s in Zimbabwe (Munhenga et al. 2008; Lukwa et al. 2014). During malaria eradication campaign, IRS using dichloro-diphenyl-trichloro-ethane (DDT) was one of the key methods by which malaria was eradicated in the temperate zones and in reducing malaria incidence in India from 75 million cases per annum in the 1930s to 110,000 per year in the 1960s (WHO 2010).

The efficacy of IRS as a vector control tool depends strongly on the behaviour of the vector mosquito, and is more effective against vectors which rest indoors (endophily). This characteristic is typically observed for the major malaria vectors in sub-Saharan Africa and has contributed greatly to the success of IRS as one of the leading malaria control strategies in this region (Pates and Curtis 2005, O’Meara et al. 2010). However, most of the mosquito species which are in close contact with people and domestic animals are usually found resting inside houses or in animal shelters, therefore giving the impression that these are their only resting places (Bhatt et al. 1989). The resting behaviour of vectors could be much more variable, with some species expressing tendency to rest outdoors (exophily), indoors (endophily), either on sprayable locations (walls and roofs) or unsprayable surfaces (household objects). The tendencies by vector mosquitoes to rest outdoors and on unsprayable locations inside houses
limit the effectiveness of IRS as a strategy for malaria prevention (Durnez and Coosemans 2013).

For the past five years, indoor spraying of roofs higher than 3.5 m from the floor level was rarely accomplished in Burma Valley and Zindi villages in Mutare and Mutasa districts respectively, of Manicaland province in Zimbabwe, following non provision of spray extension lances by the National Malaria Control Programme (NMCP). Spray extension lances enable spraying of surfaces not usually accessible with standard lances provided as components of the sprayers. The unsprayed surfaces provide alternative resting places for vectors in these sprayed structures, thus undermining the effectiveness of IRS in fighting against malaria transmission.

The resting behaviour of mosquitoes may vary considerably between different species, and even in the same species in different areas and seasons (Bhatt et al. 1989). However, behavioural change by malaria vector mosquitoes, so that a larger proportion of the species rests outdoors or indoors on unsprayed surfaces in sprayed structures, threatens to reverse the gains made by IRS in malaria control (Durnez and Coosemans 2013).

Although An. funestus and An. gambiae s.s. are naturally endophilic species in Africa, behavioural resistance in vectors in some countries has arisen in response to prolonged spraying programmes, especially using DDT and/or pyrethroids (Pates and Curtis 2005). This may result from an immediate response to the irritant and/or repellent insecticides, particularly DDT or pyrethroids, or it may be a genetic trait evolved selection pressure from the presence of insecticides in the houses (Pates and Curtis 2005). The irritant effect of insecticide was demonstrated in Burkina Faso where a 94% exit rate of An. funestus and An. gambiae from pyrethroid-treated huts was observed (Darriet 1991).

The response of the vector mosquitoes to prolonged IRS programme in Burma Valley and Zindi areas in Mutare and Mutasa districts respectively is little known. Despite the number of studies on resting behaviour of malaria vector mosquitoes in Zimbabwe (Masendu 1996, Dandalo 2007), those that looked at their resting behaviour in Mutare and Mutasa districts are scarce. Most of the researchers on malaria and vectors have concentrated in Gokwe district in the Midlands.
province, Zimbabwe. Information on the resting behaviour of anopheline mosquitoes and its relationship to major vector control tools is of great importance in malaria prevention and control. The present work aimed at providing detailed information on the resting behaviour of the adult female Anopheles mosquito, following decades of IRS implementation in Mutare and Mutasa districts, Zimbabwe. This will help to improve on future application of appropriate modes of malaria control strategies and maximize the use of ever dwindling resources.

**Materials and Methods**

**Study sites**

The study was conducted in the villages of Burma Valley (19°11’ S, 32°48’ E), Mutare district, and Zindi (18°22’ S, 32°56’ E), Mutasa district in Manicaland province, Zimbabwe. Both sites have one rainy season, which begins in November and ends in March. The sites have two distinct settlement patterns. The greater pattern is the rural set up, with housing sites which are randomly located, while the smaller part comprises farming compounds, with linear housing settlements. Both settlement patterns have similar dwellings which are characterized by brick and mortar walls under either asbestos or iron sheets roofs or pole and mud walls under grass-thatched roofs. Settlement and housing patterns are important elements in determining the resting behaviour of Anopheles mosquitoes. Malaria which is a public health concern is considered seasonal in the two study sites and is usually most prevalent during the rainy season (Sande et al. 2015). Indoor residual spraying and LLINs are the most important vector control tools employed in the two sites to reduce malaria transmission.

**Mosquito collection**

Indoor and outdoor resting mosquitoes were collected at intervals of two days per month per site for one year between January and December 2014 using prokopac battery powered aspirator (Vazquez-Prokopec et al. 2009), pyrethrum spray catch (PSC), exit window trap (EWT), and artificial pit shelter (PS) methods (WHO 2003). During each collection day, PSC and prokopac collections were performed on Monday mornings in Burma Valley and Wednesday mornings in
Zindi, while EWT and PS catches were on Tuesday mornings in Burma Valley and Thursday mornings in Zindi. Pyrethrum spray catch and prokopac methods collected indoor resting mosquitoes, while outdoor resting specimens were sampled by artificial pit shelter. Exit trap was used to catch mosquito species which enter houses at night, bite and leave soon after feeding without resting indoors as well as gravid mosquitoes leaving dwellings for oviposition (Pates and Curtis 2005, Fornadel and Norris 2008).

Indoor resting mosquito collection by PSC method was performed in 10 purposively sampled bedrooms in each site. The sampled rooms were neither sprayed nor issued with LLINs, and were visited between 06:00 and 10:00 hours for each sampling day. The PSC involved removing large furniture items, completely covering the floor and other small household goods with white clothing materials. Insecticide spraying commenced from the outside onto the eaves and doors to drive mosquitoes inside, completing the activity by spraying the entire inside of the room. All doors and windows remained closed for 10 minutes before collection of knocked down mosquitoes. A pyrethroid-insecticide aerosol (commercially marketed as Baygon) with Piperonyl butoxide synergist which inhibits oxidase activity was used. A synergist is a product which does not itself have insecticidal properties, but which, when mixed or applied with insecticides of a particular class, considerably enhance their potency by inhibiting an enzyme that normally acts to detoxify the insecticide (WHO 2013).

The percentages of adult mosquitoes collected by PSC were compared with those collected by prokopac method. Mosquito sampling by prokopac also targeted 10 bedrooms in each site, and the selected structures were unsprayed and had not been issued with LLINs. The houses were purposively selected to maximise production, and collection was conducted between 06:00 and 10:00 hours. Mosquito sampling using prokopac was carried out systematically following resting locations inside houses (Fig. 1). The resting surfaces were categorized into sprayable locations (walls, roofs) and unsprayable locations (household furniture and other objects). Samples collected were classified into four groups: wall, roof, furniture and other household goods capture stations. Mosquito samples collected on the walls were further classified into specific heights above the floor: low (< 1 m), middle (1-1.5 m), and high (>1.5 m), with specimens recorded accordingly.
Fig. 1. Mosquito collection from different resting locations using prokopac aspirator. (A) Wall. (B) Roof. (C) Furniture. (D) Other household goods (Hanging clothing).

Adult mosquitoes leaving the houses after successful or unsuccessful attempts to feed, and those which exited dwellings to lay eggs were collected by EWTs fixed on broken window panes in five sprayed and five unsprayed bedrooms (10 bedrooms) without LLINs at each site following WHO standards (1975). The traps were installed only to houses without large spaces under the eaves, and in areas where dwellings with such specifications were not available; the large spaces were reduced by fixing cotton wool. The EWTs were set between 16:00 and 18:00 hours, with mosquitoes caught aspirated between 06:00 and 10:00 hours the following morning.

Outdoor resting mosquito species were collected from 10 artificial pit shelters (WHO 1975), at each site, conveniently located in the villages of the two study areas. In each site, five pit shelters were located near human dwellings, while the remaining five were sited close to cattle shelters. Artificial pit shelters were dug in shaded places under trees or thick bushes. Each was rectangular in shape, 1.5-1.8 m deep, 1.2-1.5 m long and 0.9-1.2 m wide. In each of the four vertical sides, about 45-60 cm from the bottom of the pit, a small cavity of 15 cm width and 30 cm depth was dug at right angles to the vertical wall. These cavities have been reported to be most attractive resting sites for mosquitoes entering the PS (WHO 1975, Bhatt et al. 1989).
Mosquitoes collected by each method were identified using morphological keys (Gillies and De Meillon 1968), sorted, counted and recorded. All blood-fed and gravid mosquitoes collected by EWTs were examined under x 20 Zeiss light microscope to confirm their blood digestion stages. The *An. funestus* group and *An. gambiae* complex were placed individually into the eppendorf tubes with silica gel to keep them dry, and transported to National Institute of Health Research (NIHR) laboratory in Harare for sibling species identification using polymerase chain reaction (PCR).

**Species identification**

Polymerase chain reaction species identification was performed using DNA extracted from legs or wings of a few specimens selected randomly from the lot previously identified using morphological characters following the methods of Koekemoer et al. (2002) in the *An. funestus* group and Scott et al. (1993) in *An. gambiae s.l.* mosquitoes.

**Data analysis**

In this mostly descriptive study, data on mosquito species resting densities were presented in tables and charts made of proportions and percentages of the total number of *Anopheles* collected at 95% confidence intervals. Differences in mosquito resting locations and wall heights as well as sprayed and unsprayed structures between the two sites were subjected to statistical analysis using analysis of variance (ANOVA) at the 0.05 level of significance.

**Ethics**

Permission to carry out the study was granted by Manicaland provincial health authorities and respective village leadership. Both verbal and written informed consent was obtained from the head of each household selected before PSC, EWT and prokopac collections were conducted, and confidentiality was maintained.
Results

Indoor and outdoor resting behaviour of *Anopheles* mosquitoes

The relative monthly proportions of anopheline mosquitoes resting indoors and outdoors are shown in Table 1. A total of 592 indoor and outdoor *Anopheles* mosquitoes were sampled from Burma Valley and Zindi sites, and *An. funestus* group was the predominant species (96.8%, n=573), whereas *An. gambiae* complex mosquitoes were few (3.2%, n=18) of the total *Anopheles* mosquitoes collected. The results showed distinct monthly variation in indoor and outdoor resting behaviour of *An. funestus* populations and *An. gambiae s.l.* in the two areas. The *An. funestus* group and *An. gambiae s.l.* had greater endophilic than exophilic tendencies. Of note, the results revealed that combined *An. funestus* populations and *An. gambiae s.l.* collected by PSC were approximately five times greater than those caught by artificial PS. However, there were statistical differences between indoor and outdoor resting tendencies in *An. funestus* from January to April, but no differences in the two behaviours from September through to December (Table 1). There were neither indoor nor outdoor collections made during mid-winter (June and July).
Table 1 Percentage of *Anopheles* mosquitoes caught resting indoors and outdoors in Burma Valley and Zindi areas using two collection methods, Jan-Dec 2014

| Month | An. *funestus* group | | | An. *gambiae* s.l. | | |
|-------|---------------------|-----|-----------------|-----------------|
|       | N | Indoor (%) | Outdoor (%) | P-Value | N | Indoor (%) | Outdoor (%) | P-Value |
| Jan   | 75 | 86.7 | 13.3 | *0.0017 | 6 | 87.5 | 12.5 | 0.1244 |
| Feb   | 90 | 82.2 | 17.8 | *0.0004 | 4 | 100 | 0 | 0.1333 |
| Mar   | 154 | 77.9 | 22.1 | *0.0008 | 6 | 90 | 10 | 0.1328 |
| Apr   | 136 | 83.8 | 16.2 | *0.0001 | 3 | 100 | 0 | 0.1211 |
| May   | 33 | 97 | 3 | 0.1868 | 0 | 0 | 0 | - |
| Jun   | 0 | 0 | 0 | - | 0 | 0 | 0 | - |
| Jul   | 0 | 0 | 0 | - | 0 | 0 | 0 | - |
| Aug   | 0 | 0 | 0 | - | 0 | 0 | 0 | - |
| Sept  | 22 | 100 | 0 | 0.4594 | 0 | 0 | 0 | - |
| Oct   | 27 | 88.9 | 11.1 | 0.2985 | 0 | 0 | 0 | - |
| Nov   | 26 | 84.6 | 15.4 | 0.3177 | 0 | 0 | 0 | - |
| Dec   | 10 | 90 | 10 | 0.2029 | 0 | 0 | 0 | - |
| Total | 573 | 84.1 | 15.9 | *0.0009 | 19 | 89.5 | 10.5 | 0.0676 |

*, significant at p˂0.05; PSC, Pyrethrum spray catch; PS, Pit shelter

Prokopac versus PSC mosquito collection method

The proportions of mosquitoes caught in wet and dry seasons by each collection technique are depicted in Table 2. Of the 1,179 mosquitoes captured in both study areas, prokopac aspirator collection method had the most catches (60%, 709/1179) compared with PSC (40%, 470/1179). Overall, the prokopac aspirator collected two times more *An. funestus* mosquitoes per room than the PSC method. There were significant differences in the numbers of *An. funestus* adults collected by prokopac aspirator and PSC methods in wet (P= 0.02) and dry (P=0.02) seasons. However, there were no significant differences in the numbers of *An. gambiae* complex, other *Anopheles* mosquitoes and Culicines collected by the two methods in both study sites (Table 2). All the collections by prokopac aspirator were live, while PSC yielded knocked down mosquitoes. The number of *An. gambiae* s.l. and other *Anopheles* species collected was too small (10 and 9 respectively) to make a meaningful analysis.
### Table 2 Proportion of indoor resting mosquitoes collected by two techniques in Burma Valley and Zindi sites

<table>
<thead>
<tr>
<th>Species</th>
<th>Period</th>
<th>Collection method</th>
<th>Prokopac</th>
<th>P-Value</th>
<th>PSC</th>
<th>Density</th>
<th>Density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>%</td>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prokopac</td>
<td>Density</td>
<td>P-Value</td>
<td>PSC</td>
<td>Density</td>
<td></td>
</tr>
<tr>
<td>An. funestus</td>
<td>Wet</td>
<td>463</td>
<td>63.8</td>
<td>2.31</td>
<td>262</td>
<td>36.2</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>310</td>
<td>68.3</td>
<td>1.06</td>
<td>98</td>
<td>31.7</td>
<td>0.49</td>
</tr>
<tr>
<td>An. gambiae s.l.</td>
<td>Wet</td>
<td>7</td>
<td>59.6</td>
<td>0.04</td>
<td>4</td>
<td>40.4</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>3</td>
<td>61.2</td>
<td>0.02</td>
<td>2</td>
<td>38.8</td>
<td>0.01</td>
</tr>
<tr>
<td>Other Anopheles</td>
<td>Wet</td>
<td>9</td>
<td>70.7</td>
<td>0.05</td>
<td>4</td>
<td>29.3</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>2</td>
<td>66.4</td>
<td>0.01</td>
<td>1</td>
<td>33.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Culicines</td>
<td>Wet</td>
<td>47</td>
<td>60.5</td>
<td>0.24</td>
<td>31</td>
<td>39.5</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>20</td>
<td>58.1</td>
<td>0.1</td>
<td>14</td>
<td>41.9</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*Wet, Nov-Mar, Dry, Apr-Oct

### Indoor resting habitats of mosquito species

Table 3 is showing variations in the resting behaviour of anopheline mosquitoes on different surfaces inside dwellings. It was observed that out of the total number of *An. funestus* mosquitoes which entered the structures to rest, nearly 90% (n=546) were collected on sprayable surfaces (walls and roofs), with about 10% (n=61) found on unsprayable surfaces (furniture and other household goods). Of the sprayable surfaces, nearly 56% (n=306) of *An. funestus* mosquitoes selected roofs as resting sites, with wall surfaces constituting about 44% (n=244) in both study sites. *Anopheles funestus* collections were 1.25 times more on the roofs than on the walls inside dwellings. A significant difference was observed in the resting habits of *An. funestus* mosquitoes on the walls and roofs inside houses (P=0.004). Among the unsprayable surfaces, more *An. funestus* and *An. gambiae s.l.* were collected resting on furniture objects than other household goods (Table 3).
Table 3 Percentages of mosquito species collected indoors according to resting surfaces

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>N</th>
<th>Wall (%)</th>
<th>Roof (%)</th>
<th>Furniture (%)</th>
<th>Other (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. funestus group</td>
<td>607</td>
<td>40.2</td>
<td>50.4</td>
<td>7.2</td>
<td>2.2</td>
</tr>
<tr>
<td>An. gambiae complex</td>
<td>31</td>
<td>35.5</td>
<td>41.9</td>
<td>16.2</td>
<td>6.5</td>
</tr>
<tr>
<td>Other Anopheles species</td>
<td>13</td>
<td>30.8</td>
<td>53.8</td>
<td>7.7</td>
<td>7.7</td>
</tr>
<tr>
<td>Culex species</td>
<td>509</td>
<td>38.3</td>
<td>47.2</td>
<td>9.4</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Wall height indoor resting preferences of mosquito species

The results showed variability in mosquito resting densities on different wall heights from the floors (Table 4). Of the An. funestus sampled, the majority (about 44%, 108/245) had greater tendencies to rest on wall heights of less than 1 m from the ground, with middle heights being least preferred resting sites (just above 20%, 54/245). Different heights of a wall above the floor was found to be a significant factor in the densities of An. funestus mosquitoes resting indoors (ANOVA: df=2; F=14.22; P=0.002). Data collected on An. gambiae s.l. mosquitoes (2.4%, 11/455) and other Anopheles species (0.9%, 4/455) over 12 months of study in both sites could not provide any meaningful interpretation.

Table 4 Percentage of indoor resting mosquito according to species and collection height above ground in Burma Valley and Zindi

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>N</th>
<th>Low (&gt;1)</th>
<th>Middle (1-1.5)</th>
<th>High (&lt;1.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. funestus group</td>
<td>245</td>
<td>44.1</td>
<td>22.0</td>
<td>33.9</td>
</tr>
<tr>
<td>An. gambiae s.l</td>
<td>11</td>
<td>63.6</td>
<td>9.1</td>
<td>27.3</td>
</tr>
<tr>
<td>Other Anopheles species</td>
<td>4</td>
<td>75.0</td>
<td>25.0</td>
<td>0</td>
</tr>
<tr>
<td>Culex species</td>
<td>195</td>
<td>39.4</td>
<td>30.3</td>
<td>30.3</td>
</tr>
</tbody>
</table>

Survival rate of exit window trap collections after 24-hour holding period

The results of Anopheles mosquitoes collected by exit window traps from sprayed and unsprayed structures in Burma Valley and Zindi sites showed variations in densities, proportions of abdominal appearance, as well as survival rate after 24-hour holding period (Table 5). The
proportion of *Anopheles* mosquitoes found dead in the exit traps were lower in unsprayed than sprayed structures. The highest proportion of *An. funestus* mosquitoes found exiting both sprayed and unsprayed dwellings were gravid. Of the gravid *An. funestus* mosquitoes caught, the majority, about 65% (204/312), were collected exiting sprayed structures in both sites. A similar trend was observed in *An. gambiae s.l.*, of which sprayed rooms had more half gravid to gravid specimens than unsprayed dwellings, though the proportion of catches for this species was low (4.2%, 30/721) for a meaningful analysis. A fairly high proportion (13%, 42/312) of fully fed *An. funestus* species was found exiting recently pyrethroid-treated structures in the two areas. There was fairly low mortality after 24-hour holding period in *Anopheles* mosquitoes collected from sprayed and unsprayed structures. The proportion of dead *An. funestus* and *An. gambiae s.l.* mosquitoes in the traps was significantly different between sprayed and unsprayed structures in both study sites (ANOVA: $df=2$; $F=103.0$; $P=0.002$), with the proportion of dead mosquitoes in sprayed structures constituting 16.7% (55/330), while unsprayed houses had 8.2% (32/391). The 24-hour survival rate in anopheline mosquitoes caught exiting sprayed and unsprayed structures was not significantly different (ANOVA: $df=3$; $F=1.07$; $P=0.48$), and the proportion of live mosquitoes after 24-hour holding period was 94.2% (259/275) for sprayed-house collections and 96.4% (346/359) for catches from unsprayed structures for both sites. *Anopheles funestus* species demonstrated endophilic habits as indicated by the ratio gravid to feed (gravid divided by fed) mosquitoes which was found to be constantly more than one.
Table 5 Proportion exit window trap catches and 24-hour survival rate of *Anopheles* mosquitoes from lambda cyhalothrin sprayed and control structures in Burma Valley and Zindi areas

<table>
<thead>
<tr>
<th>Structure</th>
<th>Mosquito species</th>
<th>N</th>
<th>Dead</th>
<th>Alive</th>
<th>Un</th>
<th>FF</th>
<th>1/2 G</th>
<th>G</th>
<th>24 hr mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprayed</td>
<td>An. funestus</td>
<td>312</td>
<td>16.3</td>
<td>83.7</td>
<td>9.0</td>
<td>65.4</td>
<td>12.2</td>
<td>13.4</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not</td>
<td>An. funestus</td>
<td>379</td>
<td>8.2</td>
<td>91.8</td>
<td>7.9</td>
<td>33.2</td>
<td>24.1</td>
<td>34.8</td>
<td>3.2</td>
</tr>
<tr>
<td>sprayed</td>
<td>group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>An. gambiae s.l.</td>
<td>18</td>
<td>27.8</td>
<td>72.2</td>
<td>11.1</td>
<td>77.8</td>
<td>11.1</td>
<td>0</td>
<td>11.1</td>
</tr>
<tr>
<td>Sprayed</td>
<td>An. gambiae s.l.</td>
<td>12</td>
<td>8.3</td>
<td>91.7</td>
<td>0</td>
<td>50.0</td>
<td>33.3</td>
<td>16.7</td>
<td>0</td>
</tr>
<tr>
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<td>An. gambiae s.l.</td>
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<td></td>
</tr>
<tr>
<td>sprayed</td>
<td>group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

(UF) Unfed, (FF) Fully fed, (½ G) Half gravid, (G) Gravid

**Density indices of *Anopheles* mosquitoes collected by pit shelter located at different distance from cattle kraals**

One pit trap in each study site collapsed in March 2014, following heavy rains and could not be rehabilitated for use throughout the study period. A total of 101 *Anopheles* mosquitoes were collected, of which 98% (99/101) belonged to the *An. funestus* group and only 2% (2/101) were *An. gambiae s.l.* Of the *An. funestus* group caught, 54.5% (54/99) were from pit shelters located near (<100 m) cattle kraals, and the remainder was collected in pits not adjacent to animal shelters in both sites. Outdoor daytime resting indices ranged from 0-1.72.

**PCR species identification**

The product of the PCR analysis showed that the 120 specimens taken randomly from morphologically identified *An. funestus* group collected by all four sampling methods (PSC, prokopac aspirator, PS and EWT) were all *An. funestus* s.s. However, all specimens morphologically identified as *An. gambiae* s.l. could not be identified to sibling species following unavailability of primers specific for this complex.
Discussion

Although *An. funestus* exhibited endophilic behaviour which was confirmed by EWTs with gravid to feed index of constantly more than one, the 16% exophilic habits shown by the results of this study, is a cause for concern to the NMCP. This behaviour is of practical importance because it makes this proportion of vector less vulnerable to IRS, consequently reducing effectiveness of IRS as a strategy to combat malaria transmission. In Iran during 1960s, when dieldrin replaced DDT, *An. stephensi* species survived the high toxicity of the insecticide through exophily habits in Zagros mountain areas (Hamon et al. 1970). Predominant endophilic mosquito populations may include varieties that exhibit exophilic habits. Probably, this tendency may be selected by the persistent use of insecticide or other human interventions, and it was considered the most likely reason for the failure of a WHO residual house spraying campaign in Garki district, Nigeria, to interrupt malaria transmission (Molineaux and Grammiccia 1980).

In Zimbabwe, there has been little effort to assemble information on resting behaviour of malaria vectors, especially *An. funestus* species. The work of Masendu (1996) observed partial exophilic behaviour in *An. gambiae s.l.* in Gokwe and Binga districts, Zimbabwe. Contrary, Dandalo (2007) reported major exophilic tendencies in *An. gambiae s.l.* and *An. merus* in Gokwe South district, Zimbabwe. Reports in Nigeria showed that *An. funestus* group and *An. gambiae s.l.* preferred to rest indoors (Oyewole et al. 2007). In Tanzania, most *An. funestus* was caught indoors (Mahande et al. 2007), and these results are consistent with the findings of this work. Indoor residual spraying is likely to be effective only if the vector mosquito concerned is endophilic, because the mosquito needs to rest on the insecticide-treated surfaces for a sufficient time for it to pick a lethal dose (Pates and Curtis 2005).

Of the endophilic *Anopheles* mosquitoes caught in this study, most were collected from the roofs/ceiling rather than the walls and other household goods. These results are slightly different to those observed in Suba district, Western Kenya, where the majority of mosquitoes were collected on the walls (Harbison et al. 2006). In Zimbabwe, it appears there are no documented studies on resting position preferences in mosquito populations of public health importance.
While indoor spraying of the walls and roofs/ceiling of houses with residual insecticides to reduce the longevity of indoor resting malaria vectors is crucial, for the past five years, NMCP in Zimbabwe has been only spraying the walls, leaving out most of the roofs/ceiling following non availability of spray extension lances to reach high resting positions. For mosquito species that rest indoors, it was generally thought that most males and females prefer to rest in the dark, low areas of the walls and therefore application of residual insecticides on the walls alone would reduce survival rates of indoor resting species to greatly reduce the chance of malaria transmission (Bidlingmayer 1994, Pates and Curtis 2005). The observations made in the present study, appear to suggest the urgent need for NMCP to procure spray extension lances, especially for Mutare and Mutasa districts to facilitate spraying of high roofs/ceiling and other high sprayable locations not usually reachable without extension lance tube.

The present work has shown that besides roofs and walls as resting locations indoors, it appears a small proportion of the *An. funestus* mosquitoes have the tendencies to rest on unsprayable locations such as furniture and other household objects. This selection of unsprayable locations for resting by *Anopheles* mosquitoes is of fundamental importance for vector control, suggesting that there might be a small proportion of persistence of malaria transmission despite high spray coverage in the study areas. The tendencies by relatively small proportion of vector mosquitoes to rest on unsprayable surfaces might be due to behavioural resistance induced by selection pressure of prolonged use of insecticides in the houses over the years.

Most wall resting *An. funestus* mosquitoes were sampled from dark areas, less than 1 m from the floor level, followed by upper wall surfaces, more than 1.5 m above the ground level and close to the roof. Observations of this study are very similar to those documented in Puerto Rico (Clark et al. 1994), Panama (Perich et al. 2000), and Trinidad (Chadee 2013). In sharp contrast, even though Harbison et al. (2006) collected a large number of mosquitoes resting at heights of less than 0.8 m in Kenya, this number was not significantly different from the number resting above that height mark. In general, it is expected to find anopheline mosquitoes resting at low heights as these species prefer to be further away from the main indoor lights, all the openings, yet close enough to positions often occupied by people (Harbison et al. 2006).
MarevangePo 2015 (unpublished data) reported WHO cone bioassay mortality of 34% in *An. gambiae s.l.* on sprayed walls at a height less than 0.5 m from the floor, while a wall height of more than 1 m of the same structure had 100% mortality in Mutasa district, Zimbabwe. Although these WHO cone bioassay results were based on four sprayed structures only, they nonetheless suggest low deposits of insecticides on the lower heights, and these are the heights which were demonstrated by the present study as being the most preferred resting location for *An. funestus* mosquitoes. Elsewhere in Africa, work by Govere et al. (2001) evaluated quality of spraying using WHO cone bioassay in Mpumalanga province, South Africa, and 100% mortality in *An. arabiensis* was observed in all cones placed on the bottom, middle and upper positions of each deltamethrin-treated wall.

Knowledge on the tendencies by mosquitoes to exit sprayed or unsprayed structures is of considerable importance in determining mosquito circulation from inside to outside and the degree of irritability as well as toxic effect on species populations leaving the treated houses (WHO 1975). In Burma Valley and Zindi areas, where IRS is a major malaria intervention tool, this work collected a larger proportion of *An. funestus* in exit traps fixed on recently pyrethroid-treated structures, suggesting a possible pyrethroid resistance to this malaria vector.

Unfed females found in exit traps on sprayed houses were probably associated with insecticide irritancy or its excito-repellent property, while those collected from unsprayed structures were most likely denied feeding by host avoidance traits (WHO 1975). A small fraction of fully blood fed females observed from sprayed and unsprayed houses in this study may be those naturally exophilic mosquito species that leave the house soon after feeding (Pates and Curtis 2005, Kulkami et al. 2006, Fornadel and Norris 2008).

High proportion of gravid mosquito collected in exit window traps in both sprayed and unsprayed structures confirmed the naturally endophilic behaviour of *An. funestus* (Pates and Curtis 2005). Additionally, these gravid mosquito collections in recently pyrethroid-sprayed houses might further suggest possibility of insecticide resistance or poor spraying techniques which calls for further studies. The possibility of insecticide resistance or poor spraying
techniques was demonstrated by high exit trap survival rates of *An. funestus* mosquitoes caught from recently pyrethroid-treated houses and kept over a 24-hour holding period.

Results of the present work agree with other studies carried out in Burkina Faso (Darriet 1991), Mexico (Loyola et al. 1991) and Tanzania (Mnzava 1995, Gerold 1997), but totally in contrast with findings in Gokwe and Binga districts, Zimbabwe, which reported more unfed *An. gambiae s.l.* mosquito collections in sprayed than unsprayed structures, with 100% species mortality regardless of spray status of the houses (Masendu 1996). More recently Dandalo (2007) collected a larger proportion of *An. gambiae s.l.* mosquitoes in exit window traps in Gokwe South district, Zimbabwe, though data were not compared between sprayed and unsprayed houses.

This work, therefore, provides evidence for the need to maximize IRS benefit in the control of *An. funestus* mosquitoes by highlighting important preferred resting locations in Burma Valley and Zindi. This information supports the need for the NMCP to pay particular attention to provision of spray extension lances to reach high roofs, improve the training of spray operators to achieve even and adequate deposition of insecticide dosages on the surfaces and to conduct further studies to investigate insecticide resistance in the two study sites.

**Acknowledgements**

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ANNEX 3

BITING BEHAVIOUR OF ANOPHELES FUNESTUS POPULATIONS IN MUTARE AND MUTASA DISTRICTS, ZIMBABWE: IMPLICATIONS FOR THE MALARIA CONTROL PROGRAMME

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8 This chapter was published as: Sande, S., Zimba, M., Chinwada, P., Masendu, H.T. and Makuwaza, A. 2016. Biting behaviour of Anopheles funestus populations in Mutare and Mutasa districts, Manicaland province, Zimbabwe: implications for the malaria control programme. *Journal of Vector Borne Diseases* 53: 118-126.
ABSTRACT

Background & objectives

Biting behaviour of Anopheles funestus in Mutare and Mutasa Districts, Zimbabwe, is little understood. An investigation was conducted to primarily compare mosquito catches indoors and outdoors, as well as blood meal sources and sporozoite rates.

Methods

Monthly adult anopheline sampling was conducted from October 2013 to September 2014 using Centers for Disease Control light traps, pyrethrum spray catch and artificial pit shelter methods. Mosquitoes sampled by light traps were divided into two cohorts. In one cohort, traps were left overnight and mosquitoes collected the following morning, while in the other set, mosquitoes were collected hourly throughout the night. Collected females were identified using morphological characters and categorised according to their abdominal status. Polymerase chain reaction was used to identify An. funestus sibling species and blood sources. Infection rate was tested by enzyme-linked immunosorbent assay.

Results

Morphological identification showed that indoor and outdoor catches comprised Anopheles funestus (98.3%) and Anopheles gambiae s.l. (1.7%). Of the 2,268 mosquitoes collected, 66.2% were caught by light traps, and 33.8% were caught resting indoors and outdoors. Anopheles funestus and An. gambiae s.l. were trapped more abundantly indoors (68%) than outdoors (32%). Both indoor and outdoor An. funestus densities were higher in wet (4.3) than dry season (1.8). In both areas, An. funestus demonstrated variable nocturnal indoor and outdoor flight activity rhythms, with two peaks during the night; between 2200-2300 hrs and 0200-0400 hrs. Human blood index was 64.3%, with Plasmodium falciparum infection rate of 1.8%.
Conclusion

The present work highlighted important information on the host-seeking behaviour, blood meal sources and infection rates. The detailed information should guide and overall improve vector control strategies.

Keywords Anopheles funestus; biting behavior; resting behavior; malaria; Zimbabwe

INTRODUCTION

Plasmodium falciparum, P. malariae and P. ovale are the human malaria parasites in Zimbabwe, of which the first constitutes 95% of all causes of morbidity and mortality\textsuperscript{1}. While Anopheles arabiensis is the principal vector of malaria parasites in Zimbabwe\textsuperscript{2}, Anopheles funestus sensu stricto (hereafter referred to as Anopheles funestus) is the primary vector in Mutare and Mutasa districts\textsuperscript{3}. Anopheles funestus which prefers to breed in permanent or semi-permanent water bodies, generally exhibits patchy and discontinuous distribution patterns, and is highly anthropophilic, with major malaria episodes in sub-Saharan Africa\textsuperscript{4}. This is more likely due to increased human-vector contact as a result of human settlements being usually located near to permanent or semi-permanent water bodies.

In Zimbabwe, indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) are the mainstays routinely applied to interrupt human-vector contact and malaria transmission. Mass and continuous distribution campaigns of LLINs have played an important role in reducing human-mosquito contact with consequent recent successes in malaria control\textsuperscript{5}. Long-lasting insecticidal nets protect against mosquito bites that mostly occur indoors at night when people are sleeping. The host-feeding activities of mosquitoes have important implications for mostly humans, since it is by this behaviour that the transmission of malaria and other vector-borne disease-causing organisms take place. Studies on host preference, indoor and outdoor host-seeking behaviour as well as biting times of mosquitoes make an important contribution to determining environment and periods of malaria transmission risk, as well as form the basis for developing methods of personal protection against bites by vector mosquitoes.
Pyrethroid resistance in mosquito vector populations in most African countries is increasingly threatening the gains made by pyrethroid-treated LLINs. In Temotu Province, the Solomon Islands, *An. farauti* was reported to avoid insecticide exposure on the nets by shifting from feeding indoors (endophagic) to outdoors (exophagic). Evidence from recent studies in Africa suggest that malaria vector mosquitoes may avoid contact with insecticide imbedded in nets by biting predominantly outdoors or early evening and/or morning. Russell *et al* documented evidence of increased proportions of outdoor feeding among malaria vector populations following scaled up insecticide-treated nets in rural Tanzania. This modification in mosquito behaviour that helps to avoid lethal effects of insecticide may result from the selection of genetically-inherited traits in response to increased coverage of LLINs and/or IRS. Such inherited traits may render LLINs and/or IRS less effective in combating malaria transmission.

While *Anopheles* mosquito feeding behaviour has been studied extensively in the Afro-tropical region, host preferences, biting rhythms and infection rates of the *An. funestus* populations remain poorly understood in Zimbabwe. There have been few documented attempts to determine blood feeding venues and seasonality, biting periodicity, host preferences as well as accurate estimation of parasite infection in Zimbabwe. These are important entomological indicators which guide vector control strategies.

In Gokwe and Binga Districts, Zimbabwe, *An. arabiensis* was found to frequently feed on humans and animals indoors and outdoors, illustrating behaviours where some mosquitoes feed on any available blood source rather than looking for the preferred host. The work by Dandalo in 2007 on *An. gambiae sensu lato* (s.l.) and in Gokwe South District in Zimbabwe showed the peak biting times to be from 2100 to 2200 hrs. Changes in mosquito biting behaviour observed in Gokwe and Binga Districts, Zimbabwe, and elsewhere in Africa might complicate studies on the determination of the feeding venues and times of vector mosquitoes as well as the ease with which LLINs can be implemented to combat malaria transmission. The behavioural characteristics of vector mosquitoes in malaria transmission may be different from region to region in Zimbabwe, and can well be understood only in the local context of available hosts, blood source preferences, behavioural resistance, and additional vector species.
Continuous monitoring and understanding behavioural responses of different vector mosquitoes to control tools is crucial to the National Malaria Control Programme (NMCP) as this facilitates the selection of the most effective control strategies. As Zimbabwe has joined the list of countries working towards eliminating malaria by 2030\(^1\), the need to understand the biological implications of increased distribution of LLINs is of paramount importance. After the scaled-up implementation of LLINs project by the NMCP in Mutare and Mutasa Districts, it is important to understand the behavioural responses of the major malaria vector, An. funestus, to these tools. The aim of this study was to characterise the host-seeking behaviour of An. funestus by determining the human blood indices, host preferences, and sporozoite infection rate. The results presented here are the first study on host-seeking behaviour of the An. funestus populations in Zimbabwe.

**MATERIALS & METHODS**

**Study sites**

Field work was conducted in the villages of Burma Valley (19°11′S, 32°48′E; elevation 679 m) and Zindi wards (18°22′S, 32°56′E; elevation 766 m) in Mutare and Mutasa Districts, respectively, of Manicaland Province, Zimbabwe. The two areas are separated by a distance of about 200 km and extend to the Zimbabwe-Mozambique border. The sites have a combined population of about 13,880 people (Burma Valley 4,506 and Zindi 9,374) whose major economic activities are agro-based. The people in the Zindi villages practice semi-commercial agriculture, with several plantations and estates that provide employment. A small number of people in Zindi are smallholder growers of coffee, tea, and banana plantations, while Burma Valley occupants mainly survive by working in tobacco farms and to a small scale, banana plantations.

The prevailing climatic conditions consist of a wet season that runs from November through to March, and a dry season spanning from April to October. Mean annual rainfall is about 800 mm, with January, February and March being the wettest months, characterized by torrential downpours in the afternoon and sometimes continuous rains for a couple of days. During the rainy season, nights and mornings are fairly warm at around 18°C. Afternoon temperatures are around 30°C with relative humidity of 70-80%, making the ecological settings in the study sites ideal for the survival of mosquitoes. The ecological features of the two sites show that they
consist mainly of open woodlands, isolated trees and grasses which frequently disappear during the dry season. Selection of the study villages was based on the ecological conditions which provide potential breeding habitats for both *An. gambiae* and *An. funestus* complexes.

Human dwellings are a mixture of mud or cement-plastered superstructures with grass-thatched roofs or asbestos/corrugated iron sheets. Domestic animals are sheltered in pens near the homesteads. Most residences are located near streams or dams or swamps which serve as suitable environments for the breeding of *An. funestus* mosquitoes. Indoor residual spraying and LLINs remain the pillars for the prevention and control of malaria in both sites, with LLIN ownership close to 100% during the 2013/14 mass distribution campaign (Marevangepo, unpublished data, 2015).

**Mosquito sampling**

Mosquitoes were sampled for one week each month over a period of one year (October 2013 to September 2014) using Centers for Disease Control (CDC) light traps (John W. Hock Ltd, Gainesville FL., USA), pyrethrum spray collection (PSC) and pit shelters (PS)\(^{13}\). During each sampling week, light trap catches were conducted on Tuesday and Thursday nights, while PSC and PS collections were done on Wednesday and Friday mornings. Sampling by light traps was divided into two cohorts. In one cohort, five indoor and five outdoor light traps were set up in the evening and left overnight and trapped mosquitoes collected the following morning. In the other set, two light traps, one indoor and the other outdoor, were set up in the evening and the trapped mosquitoes were collected hourly till sunrise of the following morning.

For both indoor and outdoor trapping, the light trap was hung about 1.5 m above the ground close to the feet of an individual sleeping under an insecticide-free mosquito net. Two teams of two people working in six-hour relays aspirated trapped mosquitoes hourly throughout the night. In the two cohorts, light trapping was conducted from 1800 to 0600 hrs for two nights per month at randomly-selected dwellings. The traps installed outdoors were set approximately 10-20 m away from houses. Catches were expressed as the mean proportion of mosquitoes collected per trap per night for overnight and mean proportion of mosquitoes per trap per hour for hourly collections. Indoor and outdoor temperature, relative humidity and rainfall patterns were
measured at hourly intervals using a digital thermometer, wet and dry bulb thermometer and rain
gauge, respectively.

Centers for Disease Control light traps were used in this study instead of the standard human
landing catch (HLC) method. The light traps were used as a proxy to approximately measure
human biting rates (HBR) of vector mosquitoes since the method has a strong correlation to
human landing catches, and three light traps would collect approximately equal numbers of
vectors as two human collectors. In addition, it was shown that where use of vector control
interventions is high and vector densities are low, CDC light traps can be used to monitor vector
HBR, especially An. arabiensis, assuming that the mosquitoes that entered a trap during any
hour, especially indoors, were those actively seeking a blood meal, and in most cases would bite
human hosts in the same hour and area if the light trap was absent.

While the golden standard method for assessing HBR is the human landing catches, its extensive
use has not been practical in many regions due to the increasing ethical and worker safety
concerns that this mosquito sampling method increases the risk of exposure of collectors to
infectious mosquitoes. In addition, the method appears to collect non-standardized data because
of variability of the attractiveness and skill of collectors.

Pyrethrum spray collection and PS methods were included mainly to determine the human blood
index (HBI) and sporozoite rate. Indoor-resting adult mosquitoes were collected by the PSC
method in 20 conveniently sampled bedrooms. On each study day, mosquitoes were collected
between 0600 to 1000 hrs from each selected bedroom. Outdoor-resting adult mosquitoes were
collected using 20 pit shelters, 10 for each site, conveniently dug in the villages of Burma Valley
and Zindi. Pits were separated from each other by a distance of not less than 200 m and located
in tree-shaded environments. Each pit shelter was 1.8 m deep, 1.5 m long and 1 m wide, with
four small horizontal cavities of 0.3 m deep dug on the walls at a height of 0.6 m from the
bottom. Mosquitoes were aspirated from each PS for two days in each month for the study
period.

Female anophelines from all collections were counted, their abdominal status determined (unfed,
partially fed, fully fed or gravid) and identified using morphological characters. Mosquitoes
belonging to the *An. funestus* group and *An. gambiae s.l.* were placed singly in labelled eppendorf tubes containing silica gel and transported to National Institute of Health Research (NIHR) laboratory in Harare for further processing.

**PCR species identification**

Polymerase chain reaction (PCR) identification of members of the *An. funestus* group was determined using DNA extracted from two legs or wings of each morphologically-identified specimen following the method described by Koekemoer *et al*17. The PCR assays were to confirm that the assays on blood meal sources and sporozoite rates were conducted only on *An. funestus s.s.* sibling species. Polymerase chain reaction species identification was conducted only to those specimens tested for blood meal sources and sporozoites.

**Blood meal sources and human blood index**

All fully blood-fed *An. funestus* mosquitoes collected were assayed using cytochrome b-based multiplex PCR for blood meal source identification18. Abdomens of fully-fed specimens were separated from the heads and thoraces, ground in 50 μL phosphate-buffered saline and analysed for blood meal sources for human, bovine, dog, goat, and pig primers. Human blood index (HBI) was calculated by dividing the number of *An. funestus* mosquitoes with human blood by the total number of *An. funestus* engorged with blood. Mixed blood meal sources were each treated as two separate blood meal sources in calculation.

**Sporozoite detection in infected mosquitoes and entomological inoculation rate**

Heads and thoraces were removed from the abdomens of dried mosquitoes and tested by enzyme-linked immunosorbent assay (ELISA) for the circumsporozoite (CS) protein antigen in the salivary glands using monoclonal antibodies specific for *P. falciparum* following the standard protocol described by Wirtz *et al*19. Tests were conducted to detect only *Plasmodium falciparum* because it is the predominant malaria parasite species in Zimbabwe, constituting 95% of all cases1. Mosquitoes were pooled and tested in groups of less than 10 according to collection method and date20,21. Enzyme-linked immunosorbent assay positive samples from the initial screening were re-tested to confirm positives and to quantify the amount of CS protein for each
sample. Sporozoite rate was obtained by dividing the number of *An. funestus* which contained *P. falciparum* sporozoites with the total number of *An. funestus* tested\textsuperscript{13}.

**Data analysis**

The indoor and outdoor human biting densities and biting times, blood meal sources and differences in sporozoite rates in *Anopheles* mosquitoes collected in the two study sites for the whole sampling period were compared using the one-way ANOVA test at 0.05 level of significance.

**Ethical issues**

The director for the Zimbabwe National Malaria Control Programme, provincial, district and village authorities as well as household owners were sensitised prior to the study and their permission sought and obtained. All hourly mosquito collectors were provided with untreated mosquito nets during each study night for the entire sampling period. Informed and free consent was obtained from hourly mosquito collectors and household owners who participated in the study. Three LLINs were donated to each participating mosquito collector and household following the study.

**Results**

**Anopheline mosquito composition and abundance**

Overall, 2,268 adult female *Anopheles* mosquitoes were collected by CDC light traps, PSC and PS methods (Table 1). All the specimens collected by the three methods fell into two major groups of *Anopheles* mosquitoes: the *An. funestus* group (98.3%) and *An. gambiae s.l.* (1.7%). Of the entire *Anopheles* mosquitoes collected, the majority were caught by CDC light traps, followed by PSC, and the least by PS. There were no significant differences in the number of *Anopheles* mosquitoes collected at the two study sites (ANOVA: df = 4; F = 2.87; P = 0.06). All the CDC mosquito collections in both sites were unfed. The PSC method had the highest proportions of fully-fed (96.0%) and gravid (98.5%) anopheline mosquitoes.
Table 1 Percentage *Anopheles* mosquito species composition by sampling method as identified by morphological means (actual numbers in parentheses)

<table>
<thead>
<tr>
<th>Site</th>
<th>Anopheles mosquitoes</th>
<th>N</th>
<th>CDC</th>
<th>PSC</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>UF</td>
<td>FF</td>
<td>G</td>
</tr>
<tr>
<td>Burma Valley</td>
<td><em>An. funestus</em> s.l</td>
<td>(1,139)</td>
<td>69.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>An. gambiae</em> s.l</td>
<td>(21)</td>
<td>71.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Zindi</td>
<td><em>An. funestus</em> s.l</td>
<td>(1,090)</td>
<td>63.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>An. gambiae</em> s.l</td>
<td>(18)</td>
<td>61.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>(2,268)</td>
<td>66.1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Indoor and outdoor catches of *Anopheles* mosquitoes

A total of 1,096 anopheline mosquitoes were collected by CDC light traps set indoors and outdoors overnight for the entire one year study period at the two sites (Table 2). The *An. funestus* group constituted the majority of the total anopheline mosquitoes collected. Of these, 68.8% were sampled indoors and the remainder was caught outdoors. The indoor and outdoor flight densities for the *An. funestus* group and *An. gambiae* s.l. were significantly different (ANOVA: df = 1; F = 12.93; P = 0.01). Comparison of flight activities for *An. funestus* between sites revealed no significant differences in mosquito numbers both indoors (ANOVA: df = 1; F = 0.02; P = 0.89) and outdoors (ANOVA: df = 1; F = 0.01; P = 0.90).

Table 2 Percentage variation of indoor and outdoor biting behaviour of *Anopheles* mosquitoes

<table>
<thead>
<tr>
<th>Site</th>
<th>An. funestus group</th>
<th>An. gambiae complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Indoor</td>
</tr>
<tr>
<td>Burma Valley</td>
<td>(588)</td>
<td>68.5%</td>
</tr>
<tr>
<td>Zindi</td>
<td>(488)</td>
<td>69.1%</td>
</tr>
<tr>
<td>Total</td>
<td>(1,076)</td>
<td>68.8%</td>
</tr>
</tbody>
</table>

‡Values in parentheses represent absolute numbers of anopheline species.
Seasonal occurrence of anopheline mosquitoes

The indoor and outdoor CDC light catches of the *An. funestus* group and *An. gambiae s.l.* varied according to the season of the year (Table 3), with approximately light trap collection densities of 3.3 and 2.0 mosquitoes per trap per night during the wet season (November to March) for *An. funestus* mosquitoes in Burma Valley and Zindi, respectively. Most of the *An. funestus* group and *An. gambiae s.l.* mosquitoes were collected during the wet season at both sites. *Anopheles funestus* indoor catches during the dry season (April to October) was lower than wet season. In general, the occurrence of *An. funestus* populations persisted from late wet to early dry season and completely absent in the mid-dry season which coincided with winter. Comparison of seasonal flight activity for *An. funestus* populations and *An. gambiae s.l.* revealed significant differences in mosquito densities sampled indoors and outdoors between seasons (wet and dry) (ANOVA: df = 4; F = 8.65; P = 0.00).
Table 3 Seasonal biting densities of indoor and outdoor CDC light trap anopheline collections from Burma Valley and Zindi sites

<table>
<thead>
<tr>
<th>Site</th>
<th>Species</th>
<th>Period</th>
<th>Indoor No. of traps</th>
<th>Density (range)</th>
<th>SD</th>
<th>Outdoor No. of traps</th>
<th>Density (range)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burma Valley</td>
<td>An. funestus</td>
<td>Wet season (Nov-Mar)</td>
<td>25</td>
<td>4.82 (3.31-6.63)</td>
<td>1.45</td>
<td>25</td>
<td>1.8 (1.12-2.43)</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dry season (Apr-Oct)</td>
<td>24</td>
<td>2.43 (0-5.92)</td>
<td>2.32</td>
<td>23</td>
<td>1.36 (0-4.71)</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>An. gambiae s.l.</td>
<td>Wet season (Nov-Mar)</td>
<td>25</td>
<td>0.16 (0-0.53)</td>
<td>0.23</td>
<td>25</td>
<td>0.1 (0-0.20)</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dry season (Apr-Oct)</td>
<td>24</td>
<td>0.09 (0-0.50)</td>
<td>0.19</td>
<td>23</td>
<td>0.03 (0-0.23)</td>
<td>0.08</td>
</tr>
<tr>
<td>Zindi</td>
<td>An. funestus</td>
<td>Wet season (Nov-Mar)</td>
<td>23</td>
<td>3.84 (2.93-5.33)</td>
<td>1.00</td>
<td>22</td>
<td>1.86 (1.01-2.73)</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dry season (Apr-Oct)</td>
<td>24</td>
<td>1.10 (0-1.82)</td>
<td>1.65</td>
<td>23</td>
<td>0.38 (0-1.52)</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>An. gambiae s.l.</td>
<td>Wet season (Nov-Mar)</td>
<td>23</td>
<td>0.52 (0-1.00)</td>
<td>0.49</td>
<td>22</td>
<td>0.10 (0-0.31)</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dry season (Apr-Oct)</td>
<td>24</td>
<td>0.46 (0-1.91)</td>
<td>0.82</td>
<td>23</td>
<td>0.08 (0-0.32)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

SD=Standard Deviation

Flight rhythm of *Anopheles funestus* group in the villages of Burma Valley and Zindi

In both study sites, the majority of adult female *An. funestus* collected indoors and outdoors exhibited flight activity almost throughout the night (Fig. 1). Indoor and outdoor mosquito flight activity commenced at 2000 hrs, but steadily increased up to 2300 hrs, and decreased either gradually or sharply thereafter to the point of almost zero flight around 0100 hrs. However, flight activity peaks varied slightly at collection sites, with two peaks: the first peak during the first six hours of the night and the second during the last six hours. From 0200 hrs, the indoor flight activity rhythm increased steadily with peak being observed between 0200 and 0300 hrs in Burma Valley and 0200 to 0400 hrs in Zindi. The major flight activity periodicity occurred during the second half of the night. The observed indoor flight activity of the *An. funestus* group
exceeded the outdoor flight activity in both sites and there were significant differences between
the indoor and outdoor flight activity (ANOVA: df = 3; F = 8.25; P < 0.01). The outdoor flight
activities of the An. funestus group were apparently similar in both areas, except for a sharp fall
after 0300 hrs in Burma Valley.

![Graph showing biting cycle of Anopheles funestus populations in Burma Valley and Zindi sites.](image)

**Figure 1** Biting cycle of *Anopheles funestus* populations in Burma Valley and Zindi sites.

**PCR analysis of the* Anopheles funestus* sibling species**

A total of 726 *An. funestus* group mosquitoes which were either fully blood-fed or gravid were
PCR-assayed for identification to sibling species. The analysis revealed that 91.4% were *An.
funestus* s.s., 4.9% *An. leesoni*, and 3.7% could not amplify, despite the successful amplification
of positive control. However, fully blood-fed and gravid *An. gambiae* s.l. specimens were not
PCR-tested to sibling species due to non-availability of primers to differentiate members of the
*An. gambiae* complex.
Identification of blood meal sources of *Anopheles funestus*

The PCR diagnostic identified 272 blood meals of humans and domestic animals from engorged *An. funestus* collected from the two study sites (Fig. 2). The results indicated that the majority of *An. funestus* fed on human blood source (anthropophily), resulting in HBI of about 64% (n = 175) for both indoor and outdoor resting mosquitoes collected at the two sites (Table 4). The remaining proportions of blood meals were received from domestic animals (zoophily). Among the animals, *An. funestus* showed fairly high preference for bovine blood, with dog blood least preferred. Only one specimen showed mixed bovine and goat blood sources.

Figure 2 PCR identified blood meal sources of *Anopheles funestus* collected from Burma Valley and Zindi areas. Lanes I and 13: 100bp molecular ladder, Lane 12: Negative control, Lane 11: Positive control human, Lane 2: Pig (453 base pair), Lanes 3, 7, 8, 9 and 10: Human (334 base pair), Lane 4: Mixed (Goat 132 and Bovine 561 base pair), Lane 6: Bovine (561 base pair), Lane 5: Dog (680 base pair).
Table 4 Proportion of blood meal sources of *Anopheles funestus* collected from Burma Valley and Zindi sites

<table>
<thead>
<tr>
<th>Site</th>
<th>Blood meal source</th>
<th>N</th>
<th>Human</th>
<th>Bovine</th>
<th>Dog</th>
<th>Goat</th>
<th>Pig</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burma Valley</td>
<td></td>
<td>(124)</td>
<td>63.7</td>
<td>13.7</td>
<td>1.6</td>
<td>16.1</td>
<td>4.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Zindi</td>
<td></td>
<td>(148)</td>
<td>64.8</td>
<td>14.9</td>
<td>1.4</td>
<td>11.5</td>
<td>7.4</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>(272)</td>
<td>64.2</td>
<td>14.3</td>
<td>1.5</td>
<td>13.8</td>
<td>5.8</td>
<td>0.4</td>
</tr>
</tbody>
</table>

† Values in parentheses denotes absolute numbers

**Detection of circumsporozoite antigen and entomological inoculation rate**

Table 5 shows the total number of *An. funestus* specimens tested for *P. falciparum* circumsporozoite and infection rates using ELISA. Only about 2% (n = 8) of the specimens tested positive for *P. falciparum* sporozoites for the mosquitoes collected from the two study sites. Partitioning the infection rates by site indicated a close to 2% sporozoite rate for Burma Valley and about 1% for Zindi.

Table 5 Number of *Anopheles funestus* tested and percentage found with *Plasmodium falciparum* circumsporozoite antigens

<table>
<thead>
<tr>
<th>Site</th>
<th>No. tested</th>
<th>No. positive</th>
<th>Sporozoite rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burma Valley</td>
<td>211</td>
<td>5</td>
<td>2.4</td>
</tr>
<tr>
<td>Zindi</td>
<td>243</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>454</strong></td>
<td><strong>8</strong></td>
<td><strong>1.8</strong></td>
</tr>
</tbody>
</table>

**DISCUSSION**

Work by Lines et al\(^{14}\) in Tanzania and Fornadel et al\(^{15}\) in Zambia demonstrated that CDC light trap is comparable to human landing catches and therefore considered a proxy tool for sampling
malaria vector mosquitoes that would otherwise feed on humans. While the light trap has been shown to underestimate the actual mosquito biting risk\textsuperscript{22}, CDC traps are important as they can be used to monitor HBRs in malaria vector mosquitoes in areas where use of vector control interventions is high, with low vector densities\textsuperscript{15}. Mosquitoes caught by CDC light traps in the present work compare with those reported in similar studies\textsuperscript{14,23} in that each trap was set next to a human and that the majority of the mosquitoes collected were unfed, suggesting that the mosquitoes were caught in the act of host-seeking.

Investigating host-seeking behaviour, host preferences and presence of sporozoites in \textit{Anopheles} mosquitoes is necessary to understanding their probability as vectors of malaria. Sharp\textsuperscript{24} demonstrated that the biting behaviour of \textit{Anopheles} mosquitoes can be markedly disrupted by changes in environmental factors during the night, especially rain and wind. Wind is known to have a direct effect on mosquito flight\textsuperscript{25,26}. However, no major adverse weather conditions were encountered during the entire period of the study and it was possible to collect mosquitoes at different venues and time, which indicated important entomological information that could be utilized to implementing appropriate vector control interventions.

In Burma Valley and Zindi, \textit{An. funestus} was found to be the major anopheline in the two areas and the species demonstrated predominantly indoor flight patterns. An estimate of the degree of endophagy and exophagy can be obtained when the relative proportions of the mosquitoes attempting to bite indoors and outdoors are compared\textsuperscript{27}. The CDC light catches in this study demonstrated that mosquitoes were more abundantly indoors (68\%) than outdoors (32\%), suggesting that the indoor nocturnal host-seeking tendencies of \textit{An. funestus} and \textit{An. gambiae s.l.} could be interrupted by the intra-domiciliary use of LLINs by the majority of residents of Burma Valley and Zindi areas. However, the relevance of outdoor host-seeking behaviour of mosquitoes to vector control might depend hugely on the coincidence between outdoor biting intensity and human outdoor activity\textsuperscript{28}.

Comparative historical indoor and outdoor biting profiles for malaria vector mosquitoes in Zimbabwe are lacking from published literature. However, the results of the present study are consistent with the previous studies in Uganda in which most \textit{An. funestus} populations and \textit{An. gambiae s.l.} fed indoors\textsuperscript{28}. The finding of this work contradicts those from other studies which
showed outdoor host-seeking profiles in *An. arabiensis* from Nigeria\textsuperscript{29}, and *An. gambiae s.s.* from Bioko Island, Equatorial Guinea\textsuperscript{30}. In other example *An. neivai* in Colombian Pacific\textsuperscript{31}, fed outdoors following exposure to insecticide pressure. Although this study showed dominance in indoor CDC light trap catches, the densities appear to be strongly dependent upon seasons (wet or dry).

In the present work, seasonal mosquito flight profiles were described and categorised into wet and dry seasons. The densities of *An. funestus* populations and *An. gambiae s.l.* were higher during the wet season than dry season which, in Zimbabwe, corresponds with the period of the year, usually February/March when vector density is generally at its peak following abundance of breeding sites, suitable temperatures and relative humidity\textsuperscript{10}.

Little is known about the seasonal host-seeking behaviour of malaria vectors in Zimbabwe for comparative purposes. However, similar results have been reported in Nigeria for *An. gambiae* but with *An. funestus* having high dry seasonal biting tendencies\textsuperscript{29}. The results of the current study suggest that while it is important to conduct mass and continuous net distribution campaigns all year round, it is critical to intensify net hang-up campaigns in wet season, but this should not preclude this activity the rest of the year.

When the indoor and outdoor components of hourly trap catches of *An. funestus* populations were examined, it was noted that the rhythm of flight activity fluctuated in a similar fashion throughout the night in both sites. Knowledge on the biting times of anopheline mosquitoes is crucial in ascertaining whether peak biting period coincides with that part of the night after the inhabitants retired to bed. An important finding in this context was the general nocturnal mosquito flight cycles.

The first flight activity peak which occurred prior to midnight suggests the possibility of continued malaria transmission despite net ownership and use as this was a period when probably a fairly small proportion of the rural population might still be out of bed. Further, the second peak was observed towards dawn, a period which might put some people at risk of mosquito bites as they might be out of bed for early morning household chores. This suggests
that the use of mosquito repellents would be effective to complement LLINs during the double peaks when some people would not be bed under LLINs.

Although Moiroux et al\textsuperscript{32} also observed two peaks of biting activity of \textit{An. funestus}, the times of the first and second peaks which were recorded between 12-midnight and 0100 hrs, and 0300 and 0400 hrs, respectively, differed from the peak times of this study. In contrast, in Masakadza area, Zimbabwe, Dandalo\textsuperscript{11} reported that biting of \textit{An. gambiae} complex mosquitoes commenced at 1900 hrs and ceased at 0500 hrs with biting peaks at 2200 hrs. In Kenya the biting of \textit{An. gambiae s.l.} gradually increased throughout the night with a peak three hours before dawn\textsuperscript{33}. However, the explanations for two peaks and the sharp fall in CDC light trap catches between midnight and 0100 hrs were unclear and could not be established from the present work.

The risk of transmission of mosquito-borne diseases to human populations depends greatly on the degree of human biting by vector mosquitoes, which in turn would be influenced by abundance, distribution, and host blood source preferences. In relation to the host blood meal sources, the degree of risk would depend largely on the anthropophilic or zoophilic profile of the vector mosquito. More than 64\% of the blood meals identified from \textit{An. funestus} collected from Burma Valley and Zindi were obtained from human host, suggesting that this population of \textit{An. funestus} is highly anthropophilic, and this tendency to feed on human blood increases vectorial capacity\textsuperscript{34}. The high HBI in the present study might be attributed to the attraction of the species to human habitats where no domestic animals are kept. Human blood index in \textit{An. funestus} species observed in this study is consistent with maintaining high levels of malaria transmission in the almost total absence of other vector species and is an important factor in the epidemiology of the disease as well as in estimating human-vector contact for determining malaria transmission intensity and planning for its control. However, large proportion of mixed human and animal blood meals widely reported in other studies in \textit{An. funestus} mosquitoes\textsuperscript{35-37}, and \textit{An. arabiensis}\textsuperscript{34,38} were not observed in the present work. However, this sharp contrast could not be clearly understood.

The sporozoite rate of about 2\% in \textit{An. funestus} in Burma Valley and Zindi specimens was low. Annual \textit{P. falciparum} infectious bites lower than 10\% sporozoite rate indicate its unstable transmission intensity\textsuperscript{39} and risk of epidemics\textsuperscript{40}. Basing on this submission, the results from the
current study suggest that malaria transmission in Burma Valley and Zindi might be unstable with possibility of spontaneous epidemics which calls for vigilant surveillance to avert unforeseeable disasters.

CONCLUSION

In Burma Valley and Zindi areas, malaria control strategies have greatly targeted intra-domiciliary vector mosquitoes largely through the provision of LLINs with net ownership of about 100% apiece. This tool has been proven effective against epidemiologically important anopheline vectors targeting prominently indoor biting behaviour. However, where human biting occurs outdoors and/or before midnight and/or towards dawn when people are not protected by LLINs, indoor-based mosquito net intervention might not be sufficient to reduce malaria incidence to a point where it is no longer a public health problem. An important finding in this context was that generally, the nocturnal mosquito flight cycles commenced at 2000 hrs, with double peaks between 2200 and 2300 hrs during the first six hours of the night, and between 0200 and 0400 hrs for the second six hours. By 0600 hrs, flight activities would have almost completely ceased. As such, it is clear from the results of this study that consistent use of nets every night all year round, use of personal protective clothing and repellents during peak mosquito densities might suppress malaria transmission. More so, the biting patterns of the An. funestus populations warrant further study.

ACKNOWLEDGEMENTS

We are grateful to provincial health authority for Manicaland province, which provided field staff during data collection. We are also grateful to National Institute of Health Research, which provided laboratory facilities.

REFERENCES


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ANNEX 4

THE EMERGENCE OF INSECTICIDE RESISTANCE IN THE MAJOR MALARIA VECTOR *ANOPHELES FUNESTUS* (DIPTERA: CULICIDAE) FROM SENTINEL SITES IN MUTARE AND MUTASA DISTRICTS, ZIMBABWE

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This chapter was published as: Sande, S., Zimba, M., Chinwada, P., Masendu, H.T., Mazando, S. and Makuwaza, A. 2015. The emergence of insecticide resistance in the major malaria vector *Anopheles funestus* (Diptera: Culicidae) from sentinel sites in Mutare and Mutasa districts, Zimbabwe. *Malaria Journal* **14**:466.
Abstract

Background
Insecticide resistance in major malaria vectors poses severe challenges for stakeholders responsible for controlling the disease. During the 2013/14 season, malaria vector sentinel sites in Mutare and Mutasa Districts, Zimbabwe, experienced high presence of gravid malaria vector mosquitoes resting indoors in recently pyrethroid-sprayed structures. Subsequently, an evaluation of insecticide resistance in *Anopheles funestus* populations, the major malaria vector, was conducted to better inform the Zimbabwe National Malaria Control Programme.

Methods
Indoor-resting mosquitoes were collected in randomly selected pyrethroid-sprayed houses around Burma Valley and Zindi sentinel sites in Mutare and Mutasa Districts, respectively, using prokopac aspirator in February 2014. *Anopheles funestus* mosquitoes were identified in the field using morphological keys and divided into two cohorts. One cohort was used immediately for WHO susceptibility tests and the other batch was transferred to the National Institute of Health Research insectary in Harare for oviposition. Susceptibility and intensity resistance assays were carried out on polymerase chain reaction-assayed, three to five days old, *An. funestus* s.s. F1 progeny females.

Results
Eight-hundred and thirty-six *An. funestus* and seven *Anopheles gambiae* complex mosquitoes were collected resting inside living structures. Wild caught females showed resistance to lambda-cyhalothrin (3.3% mortality), deltamethrin (12.9% mortality), etofenprox (9.2% mortality), and bendiocarb (11.7% mortality). F1 *An. funestus* female progeny indicated resistance to deltamethrin (14.5% mortality), lambda-cyhalothrin (6.9% mortality), etofenprox (8.3% mortality), and bendiocarb (16.8% mortality). Wild caught and female progeny were susceptible
to DDT and pirimiphos-methyl (100% mortality). Intensity resistance assay to bendiocarb was 100% mortality, while deltamethrin, lambda-cyhalothrin, and etofenprox had increased knockdown times with mortalities ranging between 66.7 and 92.7% after 24-hour exposures.

**Conclusion**

This study is the first report of pyrethroid and carbamate resistance in *An. funestus* populations from Burma Valley and Zindi areas and indicates a major threat to the gains made in malaria vector control in Zimbabwe. In view of the current extension and intensity of such resistance, there is urgent need to set up a periodic and systematic insecticide resistance-monitoring programme which will form the basis for guiding the selection of insecticides for indoor residual spraying and distribution of pyrethroid-treated mosquito nets.

**Keywords** *Anopheles funestus*, Insecticide resistance, Malaria vectors, Indoor residual spraying, Mortality
Background

Human malaria remains one of the most important public health challenges worldwide. In 2013, there were an estimated 198 million episodes of malaria and about 584,000 deaths globally [1]. Among the malaria-endemic countries in sub-Saharan Africa, malaria contributed 20-30% of the outpatient attendance in Zimbabwe, with about 1.5 million cases occurring annually over the past five years [2]. Approximately 98% of the cases are caused by *Plasmodium falciparum* transmitted primarily by *Anopheles arabiensis*, with *Anopheles gambiae sensu stricto* and *Anopheles funestus sensu stricto*, the secondary vectors in most regions of the country. Choi et al. [3] and Sande et al. [4, in press] have reported *An. funestus* as the major vector of malaria in Mutare and Mutasa Districts of Manicaland Province in Zimbabwe.

Improved diagnostic testing and a wider availability of effective medicines to treat malaria, as well as to control vectors predominantly through the use of indoor residual spraying (IRS) and long lasting insecticidal nets (LLINs), are the global key interventions for interruption of malaria transmission [5]. Several studies have shown the efficacy of IRS and LLINs in reducing malaria incidence in almost all settings [6,7].

Malaria control in Zimbabwe relies heavily on IRS and LLINs to target endophilic and endophagic vector mosquitoes, respectively. Presently, IRS and LLINs depend on the four most common, WHO-recommended, classes of insecticides: organochlorines, organophosphates, pyrethroids, and carbamates. Of these, pyrethroids account for the majority of IRS coverage worldwide and are at the moment used in treatment of all LLINs [8].
Since the 1940s, residual spraying with dichloro-diphenyl-trichloro-ethane (DDT) and more recently pyrethroids has been National Malaria Control Programme’s (NMCP) dominant/primary vector control practice in Zimbabwe. Mosquito nets traditionally played a much smaller role until the introduction of LLIN campaigns under the universal coverage goal over the past few years. When the LLIN distribution campaign began, there was no clear rationale for the balance of LLINs and IRS coverage in Zimbabwe as guided by WHO [5] recommendations. The high reliance on insecticide-based malaria control in public health, agriculture and at household levels has increased the selection pressure exerted by insecticides on malaria vectors [9]. The emergence and spread of insecticide resistance among malaria vectors has placed global control efforts at high risk.

Insecticide resistance is the ability of an insect population to survive exposure to the dosage of a given compound that is lethal to the majority of individuals of a susceptible lineage of the same species [9]. Malaria vectors are able to resist the action of insecticides due to various resistance mechanisms. Among these mechanisms: metabolic resistance, which occurs when endogenous, insecticide-detoxifying enzymes become more efficient in metabolizing the insecticide, preventing it from reaching its target in the nervous system, and target site resistance, which results from modification on the site of action in resistant strains of vectors, such that the insecticide no longer binds effectively, are the most important, although metabolic resistance is the most common [10].

Pyrethroid resistance, conferred by reduced target site sensitivity arising from a single point mutation in the sodium channel gene, at times referred to as knockdown resistance, has been
confirmed in \textit{An. gambiae s.s.} in West, Central and East Arica [11]. A study by Hunt \textit{et al.} [12] documented insecticide resistance to permethrin, deltamethrin, bendiocarb, and propoxur in \textit{An. funestus} populations collected in Likoma Island in Lake Malawi. Chanda \textit{et al.} [13] reported DDT, lambda-cyhalothrin and deltamethrin resistance in \textit{An. funestus} and \textit{An. gambiae s.s.} collected in Zambia. \textit{Anopheles funestus} collected in Mozambique and Uganda showed resistance to bendiocarb, permethrin, deltamethrin, and lambda-cyhalothrin [14,15]. In Kwazulu/Natal, South Africa, \textit{An. funestus} was found to be resistant to both pyrethroids and carbamates [16].

Despite the long history of IRS in Zimbabwe, there have been few instances when resistance has been recorded [17]. \textit{Anopheles arabiensis} resistance to benzene hexachloride was recorded in Chiredzi District [18], one relating to DDT in Gokwe [19], and more recently pyrethroid resistance in Gokwe [20]. However, there are no major published studies on insecticide resistance in \textit{An. funestus} in Zimbabwe. The first \textit{An. funestus} resistance to deltamethrin, lambda-cyhalothrin and bendiocarb was reported by Choi \textit{et al.} [3] in Mandeya ward, Mutasa District.

The lack of data on the status of insecticide resistance in \textit{An. funestus}, the presence of this vector in recently pyrethroid-sprayed houses in villages around Burma Valley, Mutare District, Zimbabwe, and nearby Zindi area in Mutasa District, and high dependency on pyrethroid-based IRS and LLINs, is a cause for concern to the Zimbabwe NMCP. This study was aimed at assessing the insecticide resistance in \textit{An. funestus} populations from Burma Valley and Zindi areas in Mutare and Mutasa Districts, respectively.
Methods

Study sites

The study was conducted in Mutare and Mutasa Districts in Manicaland Province, located east of Zimbabwe, 263 and 270 km, respectively, from Harare, and bordered to the east by Manica Province in Mozambique. The study sites were Burma Valley (19°11′ S, 32°48′ E; elevation 679 m) in Mutare District and Zindi (18°22′ S, 32°56′ E; elevation 766 m) in Mutasa District (Figure 1). Burma Valley and Zindi sites are respectively situated south and north of the city of Mutare, the provincial capital of Manicaland Province. Studies were carried out from 10-23 February, 2014. Both study sites are rural areas with a total population of 13,880 (Burma Valley 4,506 and Zindi 9,374).

Figure 1  Map showing Burma Valley and Zindi study sites, Zimbabwe.
Domestic animals such as cattle, goats, chickens, and dogs are commonly kept around dwellings inhabited by people, with pigs found in only a few households. Two types of houses are common in the study sites: traditional houses, pole and mud plastered superstructures with thatched roofs, and western-style houses built using cement mortar and burnt bricks, roofed with either corrugated iron sheets or asbestos.

Both sites have a tropical climate which is hot, with annual temperature ranging from 18-30°C in winter through to summer [21]. The rainfall pattern constitutes one season per annum which usually spans November to March, with December to February, the wettest months [21]. Most small rivers and streams in Zindi empty into perennial Pungwe River, which flows to Mozambique. Burma Valley, with several streams and perennial rivers, also runs to Mozambique. The rivers and streams form extensive stagnant water bodies and marshes during the rainy season (November to March), which are potential breeding sites for vectors of public health importance.

Cultivation on the river banks is common in the villages around the study sites. Both small and large-scale farming is practiced, with the majority of the people growing maize, yams and bananas. Eastern Highlands Estates located in Zindi ward, and a few small-scale commercial farms in Burma Valley, grow tea and tobacco, respectively. Both small and large-scale farmers usually use pyrethroids, organophosphates and carbamates to protect their crops from various types of agricultural pests. *Anopheles funestus* is the major malaria vector in Burma Valley and Zindi [4, in press], with the densities fluctuating following rainfall patterns, temperatures and relative humidity. Consequently, malaria transmission is high and occurs seasonally, with highest
cases recorded towards the end of the rainy season (March/April). IRS and LLINs are the major tools deployed to interrupt malaria transmission in the villages around the study sites.

**Collection of *Anopheles funestus* populations**

Indoor-resting adult mosquitoes were collected from houses between 06.00 and 10.00 hours using a prokopac battery-powered aspirator [22]. Live mosquitoes were identified to species level using morphological features [23,24]. Mosquitoes identified as belonging to the *An. funestus* group were divided into two cohorts and held in cages where they were fed with 10% sugar solution. One cohort was used immediately for WHO insecticide susceptibility bioassays and the other batch transferred to National Institute of Health Research (NIHR) insectary in Harare to allow for oviposition.

**Laboratory processing of mosquitoes**

Live blood-fed and gravid adult female *An. funestus* were pooled and individually isolated and allowed to lay eggs. Larvae were reared through to F1 adults under standard insectary conditions of 25-27°C and 70-80% relative humidity. Polymerase chain reaction (PCR) using two legs per mosquito was carried out following the protocol of Koekemoer [25] to confirm the sibling species of all females that laid eggs, and susceptibility and intensity resistance assays were conducted on the F1 progeny only of *An. funestus s.s.* females.
**Insecticide susceptibility tests**

Randomly selected, non-blood fed, F1 progeny (3-5 days old) and gravid wild caught samples were subjected to standard WHO susceptibility tests [26]. Standard insecticide-treated papers supplied by WHO (Malaysia) were used to test for susceptibility to 4% DDT, 0.05% deltamethrin, 0.05% lambda-cyhalothrin, 0.5% etofenprox, 0.1% bendiocarb, and 1% pirimiphos methyl. Twenty to 25 female mosquitoes were exposed in each tube. Negative controls consisted of untreated papers, impregnated with different oil according to the insecticide used. Knockdowns were recorded 10, 15, 20, 30, 40 min through to one hour after the start of exposure. Final mortality was scored 24 hours post exposure and a 10% sugar solution was provided to survivors. Where the mortality in the control group was above 5% but less than 20%, correction of mortality was made by applying Abbott’s formula, with the test results discarded when control mortality was more than 20%. Results were accepted if no mortality was observed in the control. WHO [26] criterion for interpretation of results was followed for considering vector species susceptible (mortality 98-100%), potentially resistant (mortality 90-97%) and resistant (mortality <90%).

**Resistance intensity assays**

F1 progeny female mosquitoes were exposed to 0.05% lambda-cyhalothrin, 0.05% deltamethrin, 0.5% etofenprox, and 0.1% bendiocarb-treated papers continuously for eight hours with knockdown being recorded at 5-, 10-, 15-, 20-, 30-, 40-, 50-, 60-, 80-, and 120-min intervals, and hourly thereafter up to eight hours. The eight-hour cut-off was purposely selected as the likely
time a mosquito might come into contact with a sprayed wall/surface before or after a taking blood meal [3].

**Data analysis**

WHO [26] guideline for evaluating susceptibility in mosquito populations was followed in which mortality of 98-100% indicates susceptibility; 90-97% suggests potential resistance that needs to be confirmed, and less than 90% indicates resistance. Data for the two study sites were tested using two-factor without replication Analysis of Variance (ANOVA), at 5% level of significance.

**Ethical consideration**

Verbal informed consent was obtained from community leaders and each head of household or representative before mosquito collection was conducted in the selected houses.

**Results**

**Mosquito collection**

A total of 846 *Anopheles* mosquitoes were collected resting inside recently pyrethroid-sprayed houses in the villages surrounding Burma Valley and Zindi over a two-week period in February 2014. Eight-hundred and thirty-six were identified morphologically as belonging to the *An. funestus* group, seven to the *An. gambiae s.l.* and the remaining three to other *Anopheles* species.
Of the *An. funestus* group, 390 live mosquitoes were transported to NIHR for oviposition and PCR-based species identification, while 446 wild *An. funestus* female of unknown age were tested for insecticide resistance at the field insectaries with no temperature and relative humidity control. The results of these tests are summarized on Table 1. The wild-caught *An. funestus* group showed evidence of pyrethroid and carbamate resistance, but were susceptible to DDT and organophosphates. However, the sample size of wild *An. gambiae s.l.* females was too small (n = 7) to conduct meaningful susceptibility/resistance tests.

Table 1 Percentage mortality observed in WHO susceptibility tests carried out on wild caught members of the *Anopheles funestus* group in Burma Valley and Zindi, Zimbabwe

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Site</th>
<th>24 hours post exposure</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>% mortality</td>
<td>Status</td>
</tr>
<tr>
<td>0.05% Lambda-cyhalothrin (pyrethroid)</td>
<td>Burma Valley</td>
<td>47</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>P value (between sites)</td>
<td>-</td>
<td>0.35</td>
</tr>
<tr>
<td>0.05% Deltamethrin (pyrethroid)</td>
<td>Burma Valley</td>
<td>31</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>*ND</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>P value (between sites)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.5% Etofenprox (pseudo-pyrethroid)</td>
<td>Burma Valley</td>
<td>33</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>39</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>P value (between sites)</td>
<td>-</td>
<td>0.21</td>
</tr>
<tr>
<td>0.1% Bendiocarb (carbamate)</td>
<td>Burma Valley</td>
<td>38</td>
<td>21.1</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>43</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>P value (between sites)</td>
<td>-</td>
<td>0.67</td>
</tr>
<tr>
<td>4% DDT (organochlorine)</td>
<td>Burma Valley</td>
<td>36</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>P value (between sites)</td>
<td>-</td>
<td>0.50</td>
</tr>
<tr>
<td>1% Pirimiphos-methyl (organophosphate)</td>
<td>Burma Valley</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>34</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>P value (between sites)</td>
<td>-</td>
<td>0.50</td>
</tr>
<tr>
<td>Control</td>
<td>Burma Valley</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>P value (between sites)</td>
<td>-</td>
<td>0.50</td>
</tr>
</tbody>
</table>

*ND, not done
Mosquito rearing and PCR-species identification

From the 390 samples transported to NIHR insectary, 220 oviposition Eppendorf tubes were set up with individual gravid *An. funestus* females, about 134 batches were obtained, and more than 1,900 F1 adult mosquitoes emerged from both sites. The results from the PCR-based assays confirmed that all the 220 females that laid eggs, as well as the 446 wild adults used in the susceptibility/resistance test, were *An. funestus s.s.*, while analysis of wild *An. gambiae s.l.* showed that *An. arabiensis* was predominant (71.4%, 5/7) followed by a non-malaria vector, *Anopheles quadriannulatus* (28.6%, 2/7).

Insecticide susceptibility assays

Table 2 presents the mean mortalities and the standard deviations of *An. funestus* F1 progeny females that originated from the villages in Burma Valley and Zindi following exposure to insecticide-treated papers. Mortality in unexposed controls from both sites was less than 5% in all experiments and no correction of test sample mortality data was therefore required. The treated papers used were assayed on a susceptible laboratory strain of *An. arabiensis* and showed 100% mortality for all specimens and replicates (n = 100 mosquitoes per insecticide). *Anopheles funestus* was resistant to lambda-cyhalothrin, deltamethrin and etofenprox (pyrethroids), and bendiocarb (carbamate), but susceptible to DDT (organochlorine) and pirimiphos-methyl (organophosphate) at both collecting sites. There was no significant difference in mortality of mosquitoes from Burma Valley and Zindi after exposure to pyrethroids (ANOVA: df = 4; \( F = 0.23; \) \( P = 0.92 \)) and to bendiocarb (ANOVA: df = 1; \( F = 0.18; \) \( P = 0.71 \)). The difference in
percentage mortality between pyrethroid and carbamate assays and sites was also not statistically significant (ANOVA: df = 1; F = 4.39; P = 0.13).

Table 2  WHO bioassay tests for resistance on 3-5 day old female F1 *Anopheles funestus* progeny from Burma Valley and Zindi carried out in February 2014

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>24 hours % observed mortality</th>
<th>Standard deviation</th>
<th>Resistance status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (‡)</td>
<td>% mortality (range)</td>
<td></td>
</tr>
<tr>
<td>0.05% lambda-cyhalothrin</td>
<td>100 (4)</td>
<td>9 (4-13.8)</td>
<td>3.5</td>
</tr>
<tr>
<td>0.05% deltamethrin</td>
<td>87 (4)</td>
<td>12.6 (10.8-14.7)</td>
<td>1.5</td>
</tr>
<tr>
<td>0.5% etofenprox</td>
<td>90 (4)</td>
<td>3.3 (1.6-4.9)</td>
<td>1.3</td>
</tr>
<tr>
<td>0.1% bendiocarb</td>
<td>98 (4)</td>
<td>25.5 (21.3-28.8)</td>
<td>2.8</td>
</tr>
<tr>
<td>4% DDT</td>
<td>100 (4)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1.0% pirimiphos methyl</td>
<td>100 (4)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Untreated control</td>
<td>129 (5)</td>
<td>0.8 (0-1.8)</td>
<td>0.9</td>
</tr>
<tr>
<td>0.05% lambda-cyhalothrin</td>
<td>107 (5)</td>
<td>4.7 (3.8-5.7)</td>
<td>0.6</td>
</tr>
<tr>
<td>0.05% deltamethrin</td>
<td>92 (4)</td>
<td>16.3 (14.0-18.4)</td>
<td>1.6</td>
</tr>
<tr>
<td>0.5% etofenprox</td>
<td>83 (4)</td>
<td>13.3 (11.8-14.9)</td>
<td>1.1</td>
</tr>
<tr>
<td>0.1% bendiocarb</td>
<td>100 (4)</td>
<td>8 (5.5-10.2)</td>
<td>2</td>
</tr>
<tr>
<td>4% DDT</td>
<td>114 (5)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1.0% pirimiphos methyl</td>
<td>96 (4)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Untreated control</td>
<td>122 (5)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

‡, number of tubes/replicates; R, resistant; S, susceptible.

**Knockdown effect of insecticide on F1 Anopheles funestus progeny females**

Common similarities were observed in KD_{50} and KD_{95} values between the pyrethroids (lambda-cyhalothrin and deltamethrin) and carbamates (bendiocarb), and between organochlorines (DDT) and organophosphates (pirimiphos-methyl) in both Burma Valley and Zindi sites (Table 3). The knockdown effects of the four classes of insecticides tested over one hour showed more rapid
knockdown rate for DDT and pirimiphos-methyl than the other two classes of insecticides (Table 3). DDT knocked down 50 and 95% of the mosquitoes from both sites within 50 and 60 min of exposure, respectively. Fifty per cent and 95% knockdown was achieved within 50 and 80 min, respectively, for mosquitoes collected from Zindi when exposed to pirimiphos-methyl. There was loss of knockdown effect on all samples from both sites when exposed for 80 min to lambda-cyhalothrin and deltamethrin and bendiocarb.

Table 3  Association between percentage 24-hour mortality and knockdown (KD) time using WHO test tubes

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Site</th>
<th>% mortality</th>
<th>KD&lt;sub&gt;50&lt;/sub&gt; (min)</th>
<th>KD&lt;sub&gt;95&lt;/sub&gt; (min)</th>
<th>Resistance status</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05% lambda-cyhalothrin</td>
<td>Burma Valley</td>
<td>9.0</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>4.7</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td>0.05% Deltamethrin</td>
<td>Burma Valley</td>
<td>12.6</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>16.3</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td>0.1% Bendiocarb</td>
<td>Burma Valley</td>
<td>25.5</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>8.0</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td>0.5% Etofenprox</td>
<td>Burma Valley</td>
<td>3.3</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>13.3</td>
<td>No KD</td>
<td>No KD</td>
<td>R</td>
</tr>
<tr>
<td>4% DDT</td>
<td>Burma Valley</td>
<td>100</td>
<td>50</td>
<td>60</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>100</td>
<td>40</td>
<td>50</td>
<td>S</td>
</tr>
<tr>
<td>1% Pirimiphos-methyl</td>
<td>Burma Valley</td>
<td>100</td>
<td>30</td>
<td>60</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>100</td>
<td>50</td>
<td>80</td>
<td>S</td>
</tr>
</tbody>
</table>

S, susceptible; R, resistant; KD, knockdown; KD<sub>50</sub>, knockdown rate for 50% of mosquitoes; KD<sub>95</sub>, knockdown rate for 95% of mosquitoes; No KD, loss of knockdown effect (<20% of mosquitoes knocked down after 1-hour exposure).
Insecticide resistance intensity in *Anopheles funestus* F1 female progeny

*Anopheles funestus* exhibited various levels of knockdown effects after eight-hour exposure to insecticides, with highest sensitivity observed in bendiocarb for the two localities (Table 4). In both areas, there was no statistically significant difference in responses among lambda-cyhalothrin, deltamethrin and etofenprox over the entire eight-hour observation period (ANOVA: df = 5; F = 2.39; P = 0.11). Although knockdown rate for deltamethrin in Burma Valley and Zindi sites were observed from 30 and 80 min, respectively, the sensitivity of the mosquitoes to the insecticide could not stretch beyond 90% knockdown effect within an eight-hour monitoring period (Figures 2 and 3). Similarly, observations on lambda-cyhalothrin and etofenprox showed percentage knockdown rate of less than 100% for the entire experimental period in both sites.
Table 4  Resistance intensity results of F1 progeny raised from female *Anopheles funestus* collected in Burma Valley and Zindi

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>Location</th>
<th>n (§)</th>
<th>KD&lt;sub&gt;50&lt;/sub&gt; (min)</th>
<th>% Knockdown after 8-hour exposure (range)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05% λ-cyhalothrin</td>
<td>Burma Valley</td>
<td>58 (3)</td>
<td>240</td>
<td>84.4 (83.7-85.4)</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>110 (5)</td>
<td>300</td>
<td>92.7 (90.2-94.2)</td>
<td>1.7</td>
</tr>
<tr>
<td>0.05% Deltamethrin</td>
<td>Burma Valley</td>
<td>100 (4)</td>
<td>300</td>
<td>90 (86.2-93.7)</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>75 (3)</td>
<td>240</td>
<td>84 (80.9-86.6)</td>
<td>2.4</td>
</tr>
<tr>
<td>0.1% Bendiocarb</td>
<td>Burma Valley</td>
<td>39 (2)</td>
<td>120</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>105 (5)</td>
<td>80</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.5% Etofenprox</td>
<td>Burma Valley</td>
<td>24 (1)</td>
<td>300</td>
<td>66.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Zindi</td>
<td>41 (2)</td>
<td>480</td>
<td>70.7 (69.8-71.6)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

§, number of tubes/replicates.

Figure 2  Insecticide intensity resistance test in *Anopheles funestus* in Burma Valley
Discussion

The status of susceptibility/resistance to lambda-cyhalothrin, deltamethrin, etofenprox, bendiocarb, DDT and pirimiphos-methyl was evaluated in *Anopheles funestus* wild populations and F1 progeny females collected from Burma Valley and Zindi in Zimbabwe. With guidance from WHO [26] protocol for characterizing insecticide resistance, where susceptibility is defined by mortality rates above 98% and resistance by mortality less than 90% 24-hours post exposure, this study provides evidence that *An. funestus* populations from both sites were resistant to pyrethroids and carbamates, but susceptible to DDT and pirimiphos-methyl. The information is important in malaria vector control operations, which in Zimbabwe are strongly dependent on the use of insecticides in IRS and LLINs.
Zimbabwe has been using DDT for both tsetse fly and malaria vector control since 1949 [20]. Currently, DDT is being applied for malaria vector control in the low veld zones of Zimbabwe (<600 m altitude), and pyrethroids, especially lambda-cyhalothrin and deltamethrin, are used interchangeably to cover the middle veld zones (600-1,200 m altitude). Continuous application of DDT and alternating lambda-cyhalothrin with deltamethrin might increase selection pressure, resulting in early loss of sensitivity in vector populations. Choi et al. [3] reported An. funestus resistance to lambda-cyhalothrin, deltamethrin and bendiocarb for the first time in the area adjacent to the Zindi collecting site, but no DDT and organophosphate-resistant populations were detected in that area. These findings are consistent with the results of the present work, which showed resistance in An. funestus to pyrethroids and bendiocarb. In both areas, there was no statistically significant difference in 24-hour mortality among lambda-cyhalothrin, deltamethrin, etofenprox, and bendiocarb (ANOVA: df=7; F=0.93; P=0.51). The detection of deltamethrin and lambda-cyhalothrin resistance in the An. funestus populations in Burma Valley and Zindi is a worrying result as these are the most common insecticides applied interchangeably by NMCP in Zimbabwe to prevent malaria transmission in the study areas.

The build-up of pyrethroid and carbamate resistance in the An. funestus populations from the two study areas is not clear. Most probably the increase in the selection pressure exerted by pyrethroids may be attributed to their continuous use in public health, agriculture and at household level to control domestic pests. Bendiocarb resistance may be mainly associated with application in agriculture, which is a major source of livelihood in both study areas. The incrimination of agricultural use of pesticides in the selection pressure against Anopheles populations has also been reported in several countries in West Africa [27,28]. Since pyrethroid
resistance has been reported to result mainly from agricultural application, it is likely that such resistance will develop regardless of the organized use of pyrethroids in properly managed malaria control programmes [29].

Results of the present work agree with other studies that reported pyrethroid and bendiocarb resistance in the *An. funestus* populations from Malawi [12], Zambia and Zimbabwe [3], Mozambique [30], and Ghana [31]. The reported occurrence of permethrin and DDT resistance in malaria vectors in Gokwe District in Zimbabwe [20] was not detected in *An. gambiae s.l.* populations from 16 sentinel sites (Burma Valley and Zindi included) in Zimbabwe following a nationwide study [17]. Resistance to pyrethroids generally confers cross-resistance to other insecticides with the same mode of action, thus limiting the alternative choices of effective insecticide [32]. The lack of cross-resistance between pyrethroids and DDT observed in this study is consistent with the work of Coetzee and Koekemoer [33], which reported that pyrethroid resistance in *An. funestus* is mostly conferred fully or partially by monooxygenases (P450) in most countries in southern Africa. Further, it appears there is no knockdown resistance (*kdr*) gene in southern African *An. funestus* to date [33], as is also clearly indicated by the observation of this work. However, cross-resistance to pyrethroids and DDT has been reported in most mosquito species of public health importance collected from other countries as a result of a *kdr* gene [34,35].

In addition to mortality, knockdown time might be a valuable tool for the early detection of reduced susceptibility, although there are no WHO standards on knockdown time specified to indicate resistance. Knockdown time has long been accepted as an indicator of susceptibility in
vector mosquitoes to insecticides. The time provides initial data on the possible involvement of \textit{kdr} gene [29], although high frequency of a resistant gene does not necessarily translate into resistance in \textit{Anopheles} populations [36].

The results of this work have demonstrated elevated knockdown time for all insecticides tested, with the increase more pronounced in pyrethroids and carbamates than DDT and organophosphate. However, there was no difference between the time required to knockdown 100\% of the mosquitoes due to DDT and pirimiphos-methyl from the two sites (ANOVA: df=3; \(F=1.91; P=0.23\)). Although this study detected no resistance to DDT and pirimiphos-methyl, the \(KD_{50}\) and \(KD_{95}\) values obtained from both sites for these two insecticides appear to be abnormally high, ranging from 30-80 min to knockdown 100\% of the specimens. These results may be an indication of future problems with the application of DDT and pirimiphos-methyl in Burma Valley and Zindi.

The high survival rates and the increased knockdown time detected in this study raise the question of whether mosquitoes are withstanding higher concentrations of insecticide or whether longer exposure times are needed. To address the latter question, a resistance intensity test was included in the current study in order to determine the strength of resistance, although it is not the standard method of measuring resistance. Currently, the standard methods of anopheline bioassays are the WHO [26] tube assay and the CDC [37] bottle assay. Although the two methods generally agree on resistance frequencies, there has been no agreement on the application of resistance intensity test as a standard tool for measuring insecticide resistance in mosquito populations.
The CDC [37] bottle bioassay method recommends the extension of diagnostic time to two hours in order to evaluate intensity of resistance, but does not give criteria for assessing resistance intensity. Within two hours of continuous exposure, *An. funestus* from both sites showed mortality of less than 40% to lambda-cyhalothrin, deltamethrin and etofenprox, with about 80% to bendiocarb, suggesting a high level of resistance to the pyrethroids. A problem with not achieving 100% knockdown after an eight-hour exposure time to all insecticides used, save for bendiocarb, might indicate serious resistance intensity. At operational level, this poses a major challenge as it is not clear whether a mosquito rests continuously for eight hours on a sprayed surface or on a treated net, taking into cognisance the repellency and irritancy properties contained in various insecticides.

**Conclusion**

Focusing on the pattern emerging from the two study sites, it is clear that *An. funestus* resistance to pyrethroids and carbamates, and susceptibility to DDT and pirimiphos-methyl, are firmly indicated. The resistance in the *An. funestus* populations detected in this study has serious implications for the current insecticide-based malaria control efforts being undertaken in the study areas. The results seem to suggest the need for urgent and effective insecticide resistance management strategies necessary for the prevention of rapid build-up of resistance across all four commonly used classes of insecticides to control vectors of public health importance.
Competing interests

The authors declare that they have no competing interests

Authors’ contributions

SS, MZ, PC, and HTM conceived the idea and designed the study. SS, HTM, SM, and AM collected data and carried out field and laboratory experiments. SS, MZ, PC, SM, HTM, and AM drafted the manuscript. All authors read and approved the final manuscript.

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References


ANNEX 5

REVIEW

A REVIEW OF NEW CHALLENGES AND PROSPECTS FOR MALARIA ELIMINATION IN MUTARE AND MUTASA DISTRICTS, ZIMBABWE

Abstract

This review outlines and discusses the new challenges in malaria control and prospects for its elimination in Mutare and Mutasa Districts, Zimbabwe. The burden of malaria has declined intensely within the past five years in most regions in Zimbabwe, including Mutare and Mutasa Districts. The nationwide malaria reduction has been primarily linked to scaled-up of vector control interventions and early diagnosis and treatment with effective antimalarial medicines. The successes recorded have prompted Zimbabwe’s National Malaria Control Programme to commit to a global health agenda of eliminating malaria in all districts in the country. However, despite the decline in malaria burden in Mutare and Mutasa Districts, there is clear evidence of new challenges including changes in vector behaviour, resistance to insecticides and antimalarial medicines, invasion of new areas by vectors, vectors in various combination of sympatry, changes in vector proportions, outdoor malaria transmission, climate change and lack of meticulousness of spray operators. These new challenges are likely to retard the shift from malaria control to elimination in Mutare and Mutasa Districts.

Keywords: Malaria, New Challenges, Malaria Vectors, Malaria Elimination

Background

Following the aborted Global Malaria Eradication campaign in the 1960s-1970s, malaria received little international attention over the subsequent years until recently [1]. After the launch of the Roll Back Malaria (RBM) programme in 1998, most countries with endemic malaria, especially in Africa, made substantial progress in their malaria control interventions. Currently, it appears commitment has greatly improved, and partnerships exist to accelerate and sustain malaria control and elimination to achieve national, regional and global malaria targets and the malaria-related Millennium Development Goals (MGDs) [2]. Malaria elimination has been defined as permanent reduction to zero incidences of locally contracted cases [3]. The malaria target under MGD 6 (halting and beginning to reverse the incidence of malaria by 2015) has been met and 55 countries are on track to reduce their malaria burden by 75% in line with the World Health Assembly’s target of 2015. Malaria mortality decreased by 47% between 2000 and
2013 globally, and by 54% in the World Health Organization (WHO) African region, with an increasing number of countries striving towards malaria elimination [4]. This progress is primarily attributed to scaled-up vector control interventions, especially indoor residual spraying (IRS) and long lasting insecticidal nets (LLINs), as well as improved malaria diagnosis and effective treatment. Implementation of malaria control strategies in Zimbabwe has not been disturbed by any political situation during the past five years.

In Zimbabwe, vector control is a central, critical component of all malaria control strategies and the use of IRS and LLINs has increased immensely over the past decade as part of an effort towards universal coverage of all populations at risk of contracting the disease. If a universal coverage and greater than 80% use of IRS and LLINs by populations at risk of malaria are attained, consolidated and maintained, malaria transmission will be significantly reduced [2]. Over the years, new challenges have emerged, complicating the goal of controlling and eliminating malaria. Despite Zimbabwe being a member of the Malaria Elimination 8 (E8) countries in the Southern Africa, the new threats and prospects for a successful shift from malaria control to elimination in Mutare and Mutasa Districts are not well understood. The article reviews work on malaria parasites, vector species composition, insecticide resistance and responses in vector mosquitoes following prolonged use of IRS and LLINs. In this review, the aim was to identify and describe common new challenges and prospects for malaria elimination in Mutare and Mutasa Districts, Zimbabwe, where substantial and constant strides have been made towards control.

**Selected districts and data collection**

Mutare (19°39'S, 32°27'E; elevation 1,063 m) and Mutasa (18°29'S, 32°50'E; elevation 912 m) Districts in Manicaland Province (Fig. 1), Zimbabwe, are selected for review as they are among some of the few areas for which historical entomological data and related information is available. The two districts are neighbouring areas situated to the eastern part of Zimbabwe, with their district administrative headquarters being separated by a distance of about 90 km. The intensity of malaria transmission in the two districts differs considerably, with Mutasa District always in the lead. In the two districts, 95% of all malaria cases are caused by *Plasmodium*
*falciparum* [5] and primarily transmitted by *Anopheles funestus sensu stricto* [6]. The disease is seasonal, but prone to sporadic epidemics, and is considered a public health problem in the two districts. Indoor residual spraying and LLINs are the major vector control strategies employed to combat malaria. In 2014, IRS protected over 80% of the population at risk of malaria in the two districts (*Mberikunashe, unpublished data*). However, the proportion of the population protected by use of mosquito nets is not clear.

![Map showing Mutare and Mutasa study sites, Zimbabwe](image)

**Fig. 1** Map showing Mutare and Mutasa study sites, Zimbabwe

Information on mosquito vector control, behaviour, and epidemiology in Zimbabwe is available from work by various researchers as well as unpublished data from sources such as the National Malaria Control Programme (NMCP), National Institute of Health Research (NIHR), national archives and academic institutions. Work by Mpofu [7], Taylor and Mutambu [8], Masendu et al.
[9] and Sande et al. [6], reported extensively on malaria species composition and relative abundance in various regions of Zimbabwe. Leeson [10], Alves and Blair [11], Mabaso et al. [12] and Munhenga [13] documented the history of vector control through use of IRS as far back as the 1940s. Masendu [14], Dandalo [15] and Sande et al. [16,17] reported on the biting and resting behaviour of vector mosquitoes from 1996 to 2016 in various parts of Zimbabwe. Elsewhere in Africa, some changes in vector behaviour including resting and biting have been attributed to prolonged use of IRS and LLINs [18].

**Malaria situation prior to the house-spraying and mosquito net era**

Prior to the implementation of IRS and/or LLINs, endemicity of malaria in Zimbabwe was shown to be markedly influenced by altitude, varying from hyperendemic in the low attitude areas (elevation less than 700 m) to hypoendemic or completely absent on the central watershed (elevation more than 1,200 m) [8,10,11]. Malaria transmission was intense, yet clearly seasonal, peaking from February to April, and the geographical distribution was more extensive, with sporadic epidemics in some areas [10,11], including Mutare and Mutasa Districts. Random malirometric surveys, especially parasite rates were carried out as pre-control strategies in selected districts [19].

**Malaria situation after the introduction of house-spraying and mosquito nets**

Over the last decade, Zimbabwe has recorded a steady annual decline in malaria morbidity, from an annual incidence of 153 cases per 1,000 populations in 2004, to 29 cases per 1,000 populations by the end of 2013. Malaria deaths decreased from approximately 3,000 in the early 2000s to about 300 people per annum in recent years [20]. A clear reduction in malaria burden was observed in the southern and central parts of Zimbabwe, with Matabeleland South Province recording malaria cases of less than 1 per 1,000 populations in 2012 [20]. From late 2012, the NMCP upgraded Matabeleland South Province from implementing malaria control activities to pre-elimination.
The reduction in malaria burden in Zimbabwe has played a pivotal role in giving confidence to politicians, policy makers, health workers and funding agencies to keeping malaria elimination high in the national agendas. All gains have coincided with widespread adoption of various malaria control strategies, especially IRS, LLINs, and early diagnosis and effective treatment. It appears the new challenge is that most of the milestones achieved in malaria control over the years are unevenly distributed and breakable in Zimbabwe, especially in Mutare and Mutasa Districts. However, from 2003 to 2013, the malaria incidence (Fig. 2), though declining, remained relatively high in Mutare (19.5%, range 4.9-62.3%) and in Mutasa (50.9%, range 11.2-88.1%) (Zimbabwe District Health Information System 2 [ZDHIS 2], unpublished data). However, malaria control interventions were enhanced from 2009 (Mberikunashe, unpublished data). Prior and following enhanced malaria control interventions, malaria incidence rates were 21.7% (range 7.9-62.3%) and 12.4% (range 4.9-21.6%) respectively in Mutare, and 59.1% (range 49.2-88.1%) and 29.2% (range 11.2-54.0%) respectively in Mutasa (Zimbabwe District Health Information System 2 [ZDHIS 2], unpublished data).
Status of malaria elimination

In 2009, a meeting was held by Ministers of Health of eight Southern African countries, the Malaria Elimination 8 (E8), in Windhoek, Namibia, to deliberate on the mechanisms and partnerships necessary for malaria elimination in their sub-region [21]. A subsequent E8 inaugural meeting was held in Maputo, Mozambique in 2010, which served as a forum for the Ministers of Health of the eight countries to coordinate efforts and assess progress made towards malaria elimination [21]. Four frontline countries (Botswana, Namibia, Swaziland and South Africa) for E8 were positioned to immediately move from malaria control to elimination, while the remaining four (Angola, Mozambique, Zambia and Zimbabwe) were expected to consolidate malaria control, supporting the frontline countries and preparing the transition to malaria elimination phase.
Resulting from the Maputo meeting, the malaria situation was assessed in all eight rural provinces of Zimbabwe following WHO guidelines [22]. The criterion for zonal classification into malaria programme phases and milestones on the path to malaria elimination was followed, with control and consolidation (slide positive rate < 5% in all fever cases), pre-elimination (< 1 case/1000 population at risk per year), elimination (0 local acquired cases), and prevention of reintroduction (WHO certification, 3 years without local transmission) [22]. The first province in Zimbabwe to implement activities under malaria pre-elimination/elimination phase was Matabeleland South in 2013. Currently, Matabeleland North, Midlands and Mashonaland West Provinces have also been promoted to implement malaria pre-elimination/elimination activities in some districts with effect from 2015. The remaining four rural provinces (Masvingo, Mashonaland Central, Mashonaland East and Manicaland) are strongly expected to continue to implement activities in the control phase, but under tight surveillance for a possible move to pre-elimination and elimination.

Parasite and vector species composition

The predominant malaria parasite species in Zimbabwe is *P. falciparum* which accounts for over 95% of malaria cases in the country [5]. The other few malaria cases are caused by *P. malariae* and *P. ovale*. The most important vector species which transmit human malaria in Africa belong to members of the *An. gambiae* complex and the *An. funestus* group. In Zimbabwe, Mpofu [7], Taylor and Mutambu [8] and Masendu et al. [9] confirmed the presence of four members of the *An. gambiae* complex: *An. gambiae s.s.* (hereafter referred to as *An. gambiae*), *An. arabiensis*, *An. merus* and *An. quadriannulatus*. More recently, Sande et al. [6] reported the sympatric occurrence of *An. arabiensis* and *An. quadriannulatus* in Mutare and Mutasa Districts, Zimbabwe. Within the *An. gambiae* complex, *An. arabiensis* and *An. gambiae* are the major human malaria vectors in sub-Saharan Africa [23].

Previous studies on the *An. funestus group* by Evans and Leeson [24] in Zimbabwe, reported the presence of *An. funestus s.s.* (hereafter referred to as *An. funestus*), *An. leesoni* and *An. confusus*. Green and Hunt [25] reported *An. funestus*, *An. parensis* and *An. aruni* in sympatry in various parts of Zimbabwe. More recently, *An. funestus* and *An. leesoni* sibling species were detected in
Mutare and Mutasa Districts [6,26]. In the *An. funestus* group, *An. funestus* is the only member that is implicated as an important vector of malaria in sub-Saharan Africa [27].

From as far back as the early1970s, *An. arabiensis* was noted to be the primary vector of malaria in Zimbabwe while *An. gambiae* and *An. funestus* are secondary vectors [8,9]. A nationwide vector distribution survey in Zimbabwe in 2005 reported the presence of *An. funestus* only at Buffalo Ranch in Chiredzi District of Masvingo Province, in the southern region of the country [9]. The scarcity of *An. funestus* was attributed to its elimination following decades of IRS. Interestingly, a 2013-2014 study on vector species composition in Mutare and Mutasa Districts showed the resurgence of *An. funestus* in the two districts [6]. The study demonstrated the shift in dominance of *An. funestus* from a secondary to a primary vector (95.4%), with *An. arabiensis* being relegated to a secondary vector (4.6%) in the two districts. In the absence of recent species composition data from other parts of Zimbabwe, the resurgence of *An. funestus* in Mutare and Mutasa could be more widespread than previously thought.

The supremacy of *An. funestus* in Mutare and Mutasa Districts is a new challenge to malaria control and elimination, primarily because it is a more efficient vector than *An. arabiensis* [28,29]. Additionally, *An. funestus* is fairly difficult to collect in its larval stage and its adaptability to field insectary and laboratory conditions is poor, leading to inconsistent entomological studies using it. Regular entomological monitoring of vector species is of paramount importance to malaria control and elimination in any setting. The predominantly indoor resting and host-seeking traits of *An. funestus* reported by Pates and Curtis [18] in various parts of Africa and Sande et al. [16,17] in Mutare and Mutasa set opportunities for its control using IRS or LLINs, with prospects of achieving the malaria elimination goal when combined with other effective malaria interventions.

**House-spraying and use of mosquito nets for malaria control**

In Zimbabwe, IRS was started as a pilot study as far back as the 1940s using dichloro-diphenyl-trichloro-ethane (DDT) and then benzene hexachloride (BHC) [11,12, 19], and is currently the mainstay of malaria vector control in the country. In 1986, following years of DDT use,
deltamethrin was evaluated in Zimbabwe in experimental huts and the residual effect was found acceptable for malaria vector control [8]. Again in 1986, micro-encapsulated deltamethrin was tried under field conditions and recommended for widespread spraying in the country [8]. Later, in 1990, lambda-cyhalothrin was tested in a small community and the residual activity was found to be comparable to deltamethrin and suitable for nationwide use.

Since the 1940s, residual spraying with DDT and more recently with pyrethroids has been the National Malaria Control Programme’s (NMCP) major vector control intervention with the aim of reducing malaria burden [30]. Over the past five years, implementation of IRS followed the WHO recommendation of achieving spray and population coverage of at least 80%. The spray coverage and the proportion of population protected from 2009 to 2014 are shown on Table 1, with above 80% spray and population protected coverage overall. This milestone, if maintained, might be an opportunity for malaria elimination in the near future for the two districts, especially adhering to the recommendation by the WHO [22] to target all villages with annual parasite index (API) of more than 5 cases per 1,000 populations per annum. However, the new challenge is that the selection criteria of villages to be sprayed in each district by the Zimbabwe’s NMCP are not based on API, but are resource-based, leaving some villages with API of >5% in Mutare and Mutasa Districts unsprayed. Hence, sporadic malaria outbreaks experienced in Mutare and Mutasa Districts in recent years have occurred in unsprayed villages with API of >5% (Mberikunashe, personal communication), posing a serious new operational challenge to malaria control and elimination. Even with high IRS coverage of above 80% for all villages with API of >5%, the poor quality of spraying is a new challenge for malaria elimination in Mutare and Mutasa Districts. The poor quality of house spraying was revealed by WHO cone bioassay mortalities in An. gambiae s.l. of 34% at a wall height <0.5 m and 100% at a wall height of >1 m of the same structure, 24-48 hrs post spraying in Mutasa Districts [16].

Moreso, part of the new challenge is with the sprayers themselves, where, in most instances, the standard compression sprayers and mode of application depend entirely on the ability and diligence of the spray operator to deliver the correct dose in the right location [31]. While the IRS programme in Mutare and Mutasa uses the WHO’s recommended compression sprayers, NMCP has not been able to consistently provide constant flow valve (CFV) for each sprayer.
over the years. The CFVs maintain a uniform application rate as the pressure in the tank falls and enhances overall efficiency of spraying [32].

Table 1 House spraying coverage and population protected in Mutare and Mutasa districts from 2009 to 2014

<table>
<thead>
<tr>
<th>Year</th>
<th>Mutare</th>
<th>Mutasa</th>
<th>Manicaland</th>
<th>Zimbabwe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% cov.</td>
<td>% pop. prot.</td>
<td>% cov.</td>
<td>% pop. prot.</td>
</tr>
<tr>
<td>2009</td>
<td>98</td>
<td>99</td>
<td>99</td>
<td>86</td>
</tr>
<tr>
<td>2010</td>
<td>95</td>
<td>97</td>
<td>92</td>
<td>95</td>
</tr>
<tr>
<td>2011</td>
<td>89</td>
<td>100</td>
<td>86</td>
<td>93</td>
</tr>
<tr>
<td>2012</td>
<td>84</td>
<td>93</td>
<td>84</td>
<td>80</td>
</tr>
<tr>
<td>2013</td>
<td>80</td>
<td>95</td>
<td>87</td>
<td>85</td>
</tr>
<tr>
<td>2014</td>
<td>96</td>
<td>84</td>
<td>86</td>
<td>92</td>
</tr>
</tbody>
</table>

% cov. = percentage coverage; % pop. Prot. = percentage population protected

To achieve the desired results in malaria control using IRS, Zimbabwe has been employing the WHO’s recommended insecticides. Dichloro-diphenyl-trichloro-ethane and BHC were used from 1945 to 1962, BHC independently in 1972 to 1973, DDT independently from 1974 to 1987, and deltamethrin and lambda-cyhalothrin from 1988 to 2000 [12]. Insecticides used for IRS from 2001 to 2013 are shown on Table 2 and it is clear that the NMCP used pyrethroids for 13 years consecutively in Mutare and Mutasa Districts. The choice of insecticide for use in IRS was primarily guided by cost and in 2014 the NMCP switched to organophosphates (pirimiphos-methyl) following the emergence of insecticide resistance in An. funestus in Mutare and Mutasa Districts [26,30]. The lack of insecticide rotation suggests unavailability or non-use of insecticide resistance management plan which is a new challenge in achieving the malaria elimination goal.
Table 2 Insecticides, formulations and amounts used in Mutare and Mutasa districts for IRS from 2001 to 2014

<table>
<thead>
<tr>
<th>Year</th>
<th>Insecticide</th>
<th>Class</th>
<th>Formulation</th>
<th>Amount used</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroids</td>
<td>10 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2002</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroids</td>
<td>10 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2003</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroids</td>
<td>10 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2004</td>
<td>Deltamethrin</td>
<td>Pyrethroids</td>
<td>5 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2005</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>10 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2006</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>10 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2007</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>10 WP</td>
<td>Not available</td>
</tr>
<tr>
<td>2008</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>10 WP</td>
<td>26,412 sachets</td>
</tr>
<tr>
<td>2009</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>10 WP</td>
<td>27,564 sachets</td>
</tr>
<tr>
<td>2010</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>5 WP</td>
<td>36,348 sachets</td>
</tr>
<tr>
<td>2011</td>
<td>Deltamethrin</td>
<td>Pyrethroid</td>
<td>5 WP</td>
<td>42,706 sachets</td>
</tr>
<tr>
<td>2012</td>
<td>Deltamethrin</td>
<td>Pyrethroid</td>
<td>5 WP</td>
<td>39,464 sachets</td>
</tr>
<tr>
<td>2013</td>
<td>Lambda-cyhalothrin</td>
<td>Pyrethroid</td>
<td>10 WP</td>
<td>36,643 sachets</td>
</tr>
<tr>
<td>2014</td>
<td>Pirimiphos-methyl</td>
<td>Organophosphate</td>
<td>300 CS</td>
<td>37,927 bottles</td>
</tr>
</tbody>
</table>

Traditionally, mosquito nets played a much lesser role than IRS until the initiation of LLIN campaigns under the universal coverage goal over the past few years. To fully implement the two vector control interventions, Zimbabwe had no clear guidance to inform provinces to balance the deployment strategies of LLINs and IRS following the WHO’s recommendation [33], especially the effectiveness of combining versus either IRS or LLINs alone, as well as the problem of introducing the second intervention as a means of compensating for the deficiencies in the implementation of the first.

Despite reports which showed more than 90% mass distribution coverage of LLINs in Mutare and Mutasa Districts (Mberikunashe, unpublished data); net utilization data could not be easily
accessed. However, utilisation of mosquito nets amongst the population at risk in Manicaland Province was 47.5% in 2012 (*Zimbabwe Malaria Indicator Survey [ZMIS], unpublished data*), 33.5% short of the WHO’s 80% coverage for impact. The low utilisation of nets in the province may suggest equally low rate of utilisation of the product in Mutare and Mutasa Districts. This poses a new challenge as the effectiveness of mosquito nets to combat malaria largely depends on their utilisation by majority of people at risk.

**Resistance to antimalarial medicines**

The real need to intensify IRS arose when the first case of chloroquine resistance was confirmed from the Zambezi Valley, Zimbabwe in 1984 [34]. Chloroquine was then the first line antimalarial medicine to treat uncomplicated malaria in Zimbabwe [35]. By 1989, chloroquine-resistant infections had been demonstrated in most parts of the endemic zones of the country, with varying types and levels of resistance [35-37].

Following confirmation of chloroquine resistance in several parts of Zimbabwe [34-37], chloroquine was replaced by a free combination of chloroquine and sulfadoxine-pyrimethamine (SP) as first line of antimalarial medicine in the early 2000s. Subsequent studies indicated rising failure of chloroquine and SP combination and these were replaced by artemesinin-based combination therapy (ACT) in 2004. The ACT antimalarials were rolled out fully in 2007/8 and are currently in use (*Zimbabwe Malaria Programme Review [ZMPR], unpublished data*). Although Mutare and Mutasa Districts have not experienced major shortages of ACT and rapid diagnostic kits (RDT) over the past few years (*Mberikunashe, unpublished data*), the continual introductions and replacements of antimalarial medicines due to parasite resistance is a new challenge threatening the efforts towards malaria control and elimination in Zimbabwe. This situation is exacerbated when the same area experiences insecticide resistance in major malaria vectors.
Status of insecticide resistance

Only four classes of insecticides are approved by the WHO to control malaria vector mosquitoes using house spraying [38]. These are pyrethroids, organochlorines, organophosphates and carbamates. At present, all the WHO-recommended LLINs [38] are treated with pyrethroids. The high dependence on pyrethroid-based malaria control has increased the selection pressure for insecticide resistance in malaria vectors. Even though insecticides have been used for a very long period in Zimbabwe, there are very few instances where resistance has been recorded [39]. Early reports of insecticide resistance in *An. arabiensis* appeared in the 1980s in Chiredzi District, south of Zimbabwe and showed BHC resistance [40]. Masendu et al. [9] and Munhenga et al. [39] reported resistance in *An. arabiensis* to DDT and permethrin from Gokwe District respectively, Zimbabwe. No further insecticide resistance was documented in Zimbabwe till recently when pyrethroid and carbamate resistance was reported in *An. funestus* in Mutare and Mutasa Districts [26,30]. Interestingly, the same studies showed that *An. funestus* populations were susceptible to both DDT (organochlorine) and pirimiphos-methyl (organophosphates).

While the emergence of insecticide resistance in *An. funestus* in Mutare and Mutasa Districts is a new challenge likely to reverse the gains made in malaria control, the lack of cross-resistance observed between pyrethroids and DDT, and carbamates and organophosphates is an opportunity for malaria control and elimination. However, following evidence of pyrethroid and carbamate resistance in *An. funestus* collected from Mutare and Mutasa Districts in 2014 [26,30], Zimbabwe’s NMCP changed insecticide used for IRS from pyrethroids to pirimiphos-methyl 300 CS (organophosphate) in the same year. Although Kanyangarara et al. [41] showed that pirimiphos-methyl had a measurable impact on malaria incidence in Mutasa District, the new challenge with the use of pirimiphos-methyl 300 CS is that the cost is comparatively high and might be unsustainable to government and malaria stakeholders, leading to possible reversal of milestones gained in malaria control.
Resting and biting behaviour of vectors *visa-vis* indoor house spraying and mosquito nets

The effectiveness of IRS and LLINs to prevent malaria transmission largely depends on resting and biting behaviours of the vectors. Indoor house spraying is effective against indoor resting mosquitoes, whereas LLINs control malaria vectors that bite indoors. Although several studies have shown the efficacy of IRS and LLINs in reducing malaria incidence in almost all settings [42-44], outdoor transmission is a new challenge to malaria control and elimination [18].

Studies in Gokwe and Binga Districts in Zimbabwe [14] showed that the principal vector *An. arabiensis* was partially exophilic, consequently, it might not be fully amenable to control by indoor application of residual insecticides, posing a new challenge to malaria control. Studies involving *An. gambiae* complex in Masakadza village, Gokwe South District in Zimbabwe [15] demonstrated predominantly exophilic tendencies of the complex, while its peak indoor biting activity occurred at 22:00 hours, coinciding with times when some people would still be awake and out of mosquito nets. The observed biting times threatens malaria control and elimination using LLINs as a major vector control intervention. However, Mosquito outdoor biting behaviour was not evaluated in this study.

Studies in Mutare and Mutasa Districts [16] established that 84% of the *An. funestus* populations were endophilic, with a lower percentage exhibiting exophilic traits (16%). Of those collected indoors, 90% were collected on sprayable habitats (walls and roofs/ceiling) and 10% on unsprayable surfaces (furniture and other household goods). Of those collected on sprayable surfaces, 56% were collected on the roofs, with 44% on the walls. For the past five years, the NMCP could not consistently spray roofs/ceiling owing to non-availability of extension lances to spray surfaces higher than 3.5 m from the ground level. Failure to spray roofs/ceiling on which the majority of mosquito species rest is a cause for concern and is a new challenge to malaria control and elimination programmes in Mutare and Mutasa Districts.

Sande et al. [17] reported trapping *An. funestus* populations and *An. gambiae s.l.* more abundantly indoors (68%) than outdoors (32%) using Centers for Disease Control and Prevention (CDC) traps, suggesting that malaria could be interrupted by LLINs if the strategy is used by the
majority of residents in Mutare and Mutasa Districts. However, the observed variable nocturnal host-seeking behaviour of An. funestus in Mutare and Mutasa Districts, with two peaks during the night; between 22:00-23:00 and 02:00-04:00 hours is a new challenge to malaria control and elimination. Both peaks suggest that malaria transmission might be maintained despite net ownership and use as this was a period when probably a fairly small proportion of the rural population might not have gone to bed yet or might have got out of bed already for early morning household chores.

Conclusion

Opportunities and critical new challenges to the ambitious goal of malaria elimination exist in Mutare and Mutasa Districts of Manicaland Province in Zimbabwe. The predominant endophilic behaviour and high indoor blood seeking traits of An. funestus, lack of cross resistance between pyrethroids and DDT, carbamates and organophosphates, as well as scaled-up malaria control interventions, especially high house-spray coverage or LLIN distribution, the existing political will, and the Zimbabwe NMCP’s commitment to E8 agenda create prospects for malaria elimination in Mutare and Mutasa Districts in the near future. However, realising the opportunities to achieve malaria elimination goal does not provide justification for ignorance to critical new challenges which have the potential to seriously retard progressing towards regional ambitious goal of malaria elimination. The emergence of resistance to antimalarial medicines and insecticides, failure to spray all villages with an API of >5%, poor spray quality in some instances, unavailability of clear guidelines on the deployment of IRS and LLINs, the use of alternatives and possible more costly insecticide in IRS to maintain the required level of vector control interventions, as well as the resurgence of one of the most efficient malaria vectors, An. funestus, non-spraying of roofs/ceiling where majority of mosquitoes prefer to rest, and possible outdoor transmission, are the new challenges threatening the milestones gained towards malaria control and elimination in Mutare and Mutasa Districts.

Evidence presented in this review suggests that selection of malaria intervention strategies in Mutare and Mutasa Districts, especially antimalarial medicines, insecticides for IRS and use of pyrethroid-based LLINs should be based on susceptibility status to antimalarials and insecticides,
as well as resting and biting behaviour of the vector mosquitoes. These aspects are important to achieve global health agenda for malaria elimination. The NMCP and stakeholders should devise an insecticide resistance management plan as part of their vector control activities. Systematic monitoring of resistance to antimalarial medicines and insecticides, and studies on malaria vector species composition, resting and biting behaviour has to be strengthened. All results on entomological monitoring surveys conducted in any region of Zimbabwe have to be rapidly and widely disseminated to pertinent government health staff, WHO and other relevant stakeholders in the field of malaria prevention, control and elimination. It is important to closely monitor outdoor transmission of malaria and the selection of malaria intervention strategies and their implementation in Mutare and Mutasa Districts in Zimbabwe should always be evidence-based.

**Abbreviations**

ACT: Artemesinin-based combination therapy; API: Annual parasite index; BHC: benzene hexachloride; CDC: Centers for Disease Control and Prevention; CFV: Constant flow valve; CS: Capsule suspension; DDT: dichloro-diphenyl-trichloro-ethane; E8: Elimination eight; IRS: Indoor residual spraying; LLINs: Long-lasting insecticidal nets; MGD: Millennium Development Goal; NIHR: National Institute of Health Research; NMCP: National Malaria Control Programme; RBM: Roll Back Malaria; RDT: Rapid diagnostic kits; SP: Sulfadoxine-pyrimethamine; ZDHIS2: Zimbabwe District Health Information System two; ZMIS: Zimbabwe Malaria Indicator Survey; MPR: Zimbabwe Malaria Programme Review.

**Ethics approval**

Permission was sought and granted by the National Malaria Control Programme Director.

**Availability of data and materials**

Information was obtained from published and unpublished data and material sources which included National Malaria Control Programme reports, Malaria Programme Review, District Health Information System and Malaria Indicator Survey.

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Competing interests

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SS, MZ, PC, and HTM were responsible for designing the manuscript, literature review and writing the paper was done by SS, MZ, PC, HTM, JM and AM. All authors read and approved the final manuscript.

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